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Purification and characterization of ubiquitin-associated and free thiol protease inhibitors from dormant Artemia embryos.

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PURIFICATION AND CHARACTERIZATION OF UBIQUITIN-ASSOCIATED AND FREE THIOL PROTEASE INHIBITORS FROM DORMANT *ARTEMIA* EMBRYOS

by

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To

my parents, husband and friends

ABSTRACT

Encysted embryos of the brine shrimp Artemia contain a mixture of thiol protease inhibitors (TPI's) which appear to be important in the regulation of a cathepsin B-like protease. These TPI's were subjected to gel filtration, anion exchange chromatography, two types of cation exchange procedures (fast protein liquid chromatography and CM-cellulose chromatography) and high performance liquid chromatography (HPLC). Cation exchange chromatography of inhibitor preparations on a Mono S column fractionated six TPI peaks. Three protein peaks from the Mono S column eluted at 0.139 M NaCl, 0.155 M NaCl and 0.164 M NaCl. The TPI proteins have molecular weights of approximately 11.5, 12.2 and 12.6 kDa by sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis (SDS-urea PAGE). Using HPLC to purify the proteins the TPI's eluted between 39% and 41% acetonitrile from a C-18 column revealing considerable hydrophobicity of each inhibitor. One protein that eluted from the Mono S column at 0.183 M NaCl showed only one peak by rechromatography on the Mono S column, but HPLC analysis revealed three additional minor peaks. The three additional protein peaks also eluted between 39% and 41% acetonitrile from the HPLC column. SDS-urea PAGE of these protease inhibitors yielded a mixture of proteins with approximate molecular weights of 4.9 and 12.3 kDa. Amino acid sequence analysis of the major protein peak showed it to be identical to human ubiquitin

(4.9 kDa). Renaturation procedures revealed that the three minor proteins possessed significant TPI activity. This suggests that extracts from dormant Artemia embryos contain both free TPI's and those associated with ubiquitin.

CM-cellulose chromatography was performed on the TPI preparations as an alternative to fast protein liquid chromatography. HPLC of CM-cellulose purified TPI preparations fractionated four TPI's free of ubiquitin. After final purification by HPLC, only two inhibitors could be renatured. The four potential TPI's had apparent molecular weights of 12.2, 12.2, 14.5 and 14.7 kDa. It appears that extracts from dormant Artemia embryos contain a mixture of free and ubiquitin-associated low molecular weight TPI's; ubiquitin may be involved in the conformational modification of TPI's in dormant Artemia embryos.

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LIST OF ABBREVIATIONS

BANA.....N-alpha-benzoyl-DL-arginine-2-naphthylamide

BSA.....bovine serum albumin

CM-cellulose.....carboxymethyl cellulose

DEAE-Sephadex (A-50)..diethylaminoethyl Sephadex

DTT.....dithiothreitol

EDTA.....ethylenediamine tetraacetate

FPLC.....fast performance liquid chromatography

GAP.....GTPase activating protein

Gp_4G.....diguanosine tetraphosphate

GTPase.....guanosine triphosphate hydrolase

HPLC.....high performance liquid chromatography

EU.....enzyme unit

ID_{50}.....inhibitor dose which gives 50% inhibition of enzyme activity

IU.....inhibitor unit

%I.....percent inhibition

kDa.....kilodalton

K_i.....inhibition constant

K_m.....Michaelis constant

LV.....lipovitellin

mM.....millimolar

mU.....milliunit

mRNP.....messenger ribonucleoprotein

pCMB.....para-chloromercuribenzoate

pI.....isoelectric point

PMSF.....phenylmethylsulfonyl fluoride

PRS.....postribosomal supernatant

SDS-urea PAGE.....sodium dodecyl sulfate-urea
polyacrylamide gel electrophoresis

STI.....soybean trypsin inhibitor

TNBS.....trinitrobenzenesulfonic acid

TFA.....trifluoroacetic acid

TPI.....thiol protease inhibitor

I. INTRODUCTION

The level of proteins within a cell is controlled by their overall rates of synthesis and degradation. In the developmental process proteases have been implicated in protein regulation, activation and degradation. Characterization of the types of proteases and their regulators, and their roles in early development, is becoming an important area of research. Intracellular protein catabolism is a highly selective process with the purpose of efficiently removing regulatory-type proteins and preventing inappropriate hydrolysis of housekeeping proteins. Proteases are known to remove defective or normal polypeptides from cells thereby controlling the concentrations of polypeptides and enzymes in cells. These activities provide the cell with varying concentrations of proteins necessary for metabolism and embryonic development. Proteases and their inhibitors play a critical role in protein metabolism (Bond and Butler, 1987, Barrett, 1986).

One protease cannot catalyze the complete breakdown of any one protein in the cell. Therefore, cellular systems contain a variety of proteases classified by the catalytic residues at their active sites. On this basis, four classes of proteases have been recognized. These include cysteine (thiol) proteases (cathepsins B, L, H), serine proteases (trypsin, chymotrypsin), aspartic proteases (cathepsin D), and metalloproteases (enkephalins). Serine proteases pos-

sess a reactive serine side chain at the active site and the mechanism of action of these enzymes occurs by the covalent binding of substrates to the serine residue. Cysteine proteases are also referred to as thiol proteases and contain a cysteine residue at the active site which is involved in a covalent complex with their respective substrates. Aspartic proteases contain two aspartic residues at their active sites and these enzymes function by an acid-base catalysis. Metalloproteases contain metal ions at the active centre. The metal ions increase the nucleophilicity of water and polarize the peptide bond to be cleaved before nucleophilic attack (Bond and Butler, 1987).

Most studies on proteases in developmental systems have concentrated on their involvement in fertilization and not on their role in intracellular protein metabolism. Proteases have been implicated in some developmental events including the activation of inactive messenger ribonucleoproteins (mRNP's) (Spirin, 1966; Slegers et al., 1989), activation of metabolically repressed ribosomes (Metafora et al., 1971), and yolk utilization (Warner and Shridhar, 1985; Perona and Vallejo, 1985; de Chaffoy de Courcelles et al., 1980).

Studies of Artemia have shown that a variety of proteases are present in the embryos and larvae including active and latent proteases and some of these enzymes have been implicated in the yolk metabolism (Perona and Vallejo, 1985; Ezquieta and Vallejo, 1985; and Warner and Shridhar,

1985), protein synthesis regulation (Warner and Shridhar, 1985; Twardowski et al., 1976; Yablonka-Reuveni and Warner, 1979), and RNA synthesis regulation (Osuna et al., 1977).

If proteases play a role in the regulation of development in invertebrates and other eukaryotes, an understanding of the types of proteases, their localization and regulation is important. Regulation of enzyme activity is very important in maintaining proper metabolism within a cell and preventing inappropriate proteolysis.

A. Cysteine (thiol) proteases and cysteine (thiol) protease inhibitors in mammalian systems

The cysteine or thiol proteases are intracellular proteases usually found in the cytosol or in lysosomes (Bond and Butler, 1987). In some pathological cases lysosomal enzymes are found in extracellular compartments. Lysosomal enzymes are released from macrophages at inflammation sites and these enzymes may damage normal tissue and proteins in the same location. Some malignant tissues secrete a cathepsin-B like protease which is thought to degrade the extracellular matrix thereby facilitating local invasion (Sloane et al., 1981). Cysteine proteases include the lysosomal cathepsins (B, L, H, N, S, M and T), the cytosolic calpains, ATP-dependent proteases, multicatalytic proteases and metal-dependent cysteine proteases (Bond and Butler, 1987).

The subcellular localization of proteases limits the action of such proteases to appropriate areas. Lysosomal proteases are constricted to an acidic environment and only catalyze the hydrolysis of proteins that enter lysosomes. Leakage of the cathepsins from lysosome results in inactivation of their respective activity by the increase in pH and by cytosolic inhibitors. Alterations in protease activities within the cell may be due to changes in the amount and or function of their inhibitors. It is now known that cellular proteases play important roles in diseases such as muscular dystrohy, diabetes, cancer and multiple sclerosis (Bond and Butler, 1987).

The increased awareness of the importance of proteases parallels an increase in the study of cysteine (thiol) protease inhibitors (TPI's). In 1957 Finkenstadt first described the isolation of a thiol protease inhibitor (TPI) from rat liver cytosol. Since this discovery, many cellular thiol protease inhibitors have been isolated from various mammalian tissues such as human epidermis (Järvinen, 1976), human spleen (Järvinen and Rinne, 1982), various rat and human tissues (Kominami et al., 1981), and rat liver (Hirado et al., 1981). There is also a class of high molecular weight thiol protease inhibitors in human serum and urine and rat serum that have sizes between 60 and 175 kDa (Wakamatsu et al., 1982; Taniguchi et al., 1981).

It has been established that mammalian cells contain at least three types of low molecular weight cysteine protease inhibitors which differ mainly in their isoelectric points (pI values). These three types of TPI's include, the acidic type with pI values between 4.7 and 5.0, the neutral type with pI values between 6.0 and 6.5, and the alkaline type with pI's between 7.0 and 7.8 (Järvinen and Rinne, 1982). Low molecular weight TPI's (11-13 kDa) share characteristics such as stability to heat and extremes in pH (Barrett, 1985). Low molecular weight TPI's in vertebrates are either competitive or non-competitive with Ki values in the range of 5×10^{-12} M to 1.2×10^{-10} M (Kominami et al., 1982). Many members of the cystatin family of inhibitors contain a hydrophobic domain in the centre of the molecule which is extensive in cystatin B. All TPI's are specific for thiol proteases; they do not inhibit proteases of other classes.

In general, most researchers agree on the classification of low molecular weight TPI's into three groups; (1) the cystatin family, (2) the kininogen family, and (3) the stefin family. There is also a class of high molecular weight kininogens isolated from human plasma (Sasaki et al., 1986). Inhibitors are placed in each category based on their amino acid sequences, isoelectric point (pH 5-7) and size. Sequence comparisons of cysteine protease inhibitors show the presence of the domain Gln-Val-Val-Ala-Gly, and this is believed to be the active site in mammalian protease

inhibitors. Most cysteine protease inhibitors are purified by affinity chromatography on Cm-papain Sepharose 4 B columns.

Researchers have identified two intracellular types of cystatins (cystatin A and cystatin B), and three secretory types (cystatin-gamma, cystatin S and cystatin C) (Katunuma and Kominami, 1986). The cystatins are small proteins with molecular weights between 11 and 13.5 kDa and contain no carbohydrate moieties (Barrett, 1985). Most cystatins are isolated in multiple molecular forms (isoforms). The cystatins are stable to extremes in pH and temperature; Green et al. (1984) showed that they are stable at pH 2, pH 12 and 80C, while chicken cystatin can be boiled (Fossum and Whitaker, 1968). Cystatins inhibit various cysteine proteases of the papain superfamily and also the exopeptidase dipeptidyl peptidase I. Cathepsins B, H, and L are also inhibited by the cystatins while calpains, calcium-dependent cysteine proteases, serine proteases and metalloproteases are not inhibited. The inhibition of proteases by cystatins has been found to be reversible and competitive with a 1:1 stoichiometry (Nicklin and Barrett, 1984).

Human cystatin S has been isolated from human saliva as an acidic inhibitor with an isoelectric point of 4.7 and a molecular weight of approximately 13.3 kDa (Isemura et al., 1986). This inhibitor has a high sequence homology with cystatin C and egg white cystatin. During the purification of cystatin S these researchers found eight proteins (S1-S8)

that cross-reacted immunologically with cystatin S. All of these proteins had molecular weights of approximately 13 kDa. Cystatin S1 had a higher isoelectric point than cystatin S while the other proteins had similar isoelectric points as cystatin S. N-terminal sequence studies showed that S4, S6 and S8 did not have residues 9, 7 and 4, respectively of cystatin S. Cystatins S5 and S7 had 4 and 3 residues, respectively at the N-terminus of cystatin S. All proteins inhibited both ficin and papain to varying extents. Both cystatins S1 and S5 had amino acid substitutions of the original cystatin S sequence. Therefore, variations in the N-terminal portion of the protein and amino acid substitutions were the cause of multiple molecular forms of Cystatin S in human saliva.

The family of kininogens has considerably higher molecular weights than those of the cystatins and stefins. Kininogen has been isolated from human plasma and chicken egg white (Sasaki et al., 1986; Kos and Turk, 1986). It inhibits calpains as well as papain-like cysteine proteases. This group of inhibitors possesses three sequence repeats that are homologous with that found in the cystatin superfamily (Müller-Esterl and Fritz, 1986).

Human stefin A has been isolated from polymorphonuclear granulocytes and is an acidic protein with an isoelectric point of 4.7 (Turk et al., 1986). The molecular weight was

found to be 11.0 kDa calculated from the amino acid sequence. Human stefin A was found to inhibit papain and cathepsins H and L.

Lenarcic et al. (1986) isolated stefin B from human spleen in two forms with isoelectric points of 5.9 and 6.5. This inhibitor also exists in an inactive dimer form (24 kDa) which can be converted to the active monomer form (12 kDa) by reduction with 2 mM dithiothreitol.

1. Regulation of protease activity

As researchers try to sort out and classify the seemingly heterogeneous but closely related family of cysteine protease inhibitors, the role of these inhibitors is becoming more clear. One role is the prevention of inappropriate proteolysis such as the regulation of lysosomal thiol protease activities in the cytosol.

Thiol protease-inhibitor complexes *in vivo* are mostly reversible, allowing the inhibitors to serve as regulatory factors to increase or decrease the concentration of free proteases (Lenney, 1980). This type of regulation would depend on the concentration of enzyme and inhibitor, the dissociation constant of the complex, and the dissociation rate of the complex. Environmental conditions such as pH or ionic strength, concentration of oxidizing agents or reducing agents may all effect the dissociation or association of enzyme-inhibitor complexes.

Indeed, cysteine protease inhibitors are becoming a dominant feature in the current literature. Also, they have become an important component in the regulation/prevention of Alzheimer's disease (Tanzi et al., 1988; Kang et al., 1988) and in the regulation of cathepsin B activity in muscle of mice with hereditary muscular dystropyhy (Gopalan and Warner, 1986). Recently protease inhibitors are implicated in the suppression of the transformation of cells by ras oncogenes (Frech et al., 1990).

Studies have found that the protein product of the Krev-1 gene, Rap 1A-p21 is a potent competitive inhibitor of GAP-mediated activation of the ras gene product, ras-p21 GTPase activity (Frech et al., 1990). The ras genes are the most common oncogenes isolated from cancer cells. Active ras genes have been found in 40% of human colon cancers. All oncogenes are mutated forms of cellular genes that regulate growth and development. Since recent findings implicate protease inhibitors in the regulation of cellular transformation by ras oncogenes (Frech et al., 1990), an understanding of the action of oncogenes may help elucidate cell regulation.

In the human genome there are at least three distinct ras genes: c-H-ras, c-K-ras and N-ras (Abraham et al., 1989). The difference between the normal ras proto-oncogenes and the transforming ras oncogenes is a single base change at one of a few positions which then results in a single amino acid substitution in ras-p21. Oncogenic ras-

p21 variants are the result of amino acid substitutions at specific positions and cause it to be mainly complexed with GTP *in vivo*. Transgenic mice that contain the activated c-H-*ras* oncogene under control of the rat elastase I regulatory element develop pancreatic tumours after a few days of pancreatic differentiation and those mice containing the normal proto-oncogene do not (Quaife et al., 1987). Neoplasia is known to occur by at least three separate mechanisms: (1) infection of a cell by a retrovirus carrying *ras* oncogenes, (2) carcinogen-induced mutation of a cellular *ras* proto-oncogene to an oncogene, and (3) insertional mutation at the regulatory element of cellular *ras* proto-oncogenes (Barbacid, 1987).

The membrane-associated *ras* proteins of relative molecular mass 21.0 kDa bind GDP and GTP and therefore are G proteins (guanine nucleotide-binding regulatory proteins). Trahey and McCormick (1987) discovered a GTPase activating protein (GAP) in the cytosol that stimulates GTPase activity of normal but not oncogenic *ras* p21. With the discovery of the GAP-*ras* protein interaction, researchers then began to examine whether GAP regulates the *ras* protein or whether the *ras* protein regulates GAP.

The G proteins including the *ras* gene product (*ras*-p21) have been implicated in the signal transduction pathway by transmitting hormonal and growth factor signals from the cell membrane to the interior of the cell (Hanely and Jackson, 1987). The G proteins are also enzymes that split off

the third phosphate of GTP producing inorganic phosphate and GDP, the latter of which remains bound to the G protein. The conversion of GTP to GDP may inactivate the G proteins (Marx, 1988).

A group of G proteins has been identified with molecular weights between 19 and 24 kDa called small G proteins. Another group of large G proteins have molecular weights between 39 and 52 kDa (Ohmori et al., 1989). The small G protein group consists of proteins encoded by the three *ras* genes (H_a- K_i- and N-) and *rho*, *ral*, *rab*2, k-*ras* and *ypt*1 genes. All of these G proteins have a consensus amino acid sequence for GTP-binding and GTPase activities (Barbacid, 1987).

Studies by various researchers implicate GAP as a regulator of *ras* action because antibodies to *ras*-p21 inhibit *ras* function and interactions with GAP (Trahey and McCormick, 1987). As well, GAP interacts with *ras*-p21 at the effector binding site (Vogel, 1988), and *ras*-p21 mutants that bind to GAP but cannot localize in the cell membrane, inhibit the function of membrane-associated *ras* proteins. Evidence suggests that *ras*-p21 has GDP-bound inactive and GTP-bound active forms and that there is a protein that converts the inactive form to the active form and a protein that is modulated by the active form (Kikuchi et al., 1989). GAP is such a protein and it directly interacts with c-*ras*-p21 proteins (Trahey and McCormick, 1987).

The Krev-1 gene product is also called rap 1A, and this product suppresses transformation of cells by the Kras oncogene. The protein product of the Krev-1 gene contains sequences for guanine nucleotide binding and shows 53% homology with the Kras gene product (Gilman, 1987; Dever et al., 1987). The amino acid sequence region that is necessary for biological activity of ras oncogenes is conserved in the Krev-1 gene product, Rap1A-p21. These results suggested that rap1A-p21 may interact with the same effector as the ras proteins and could suppress ras transformation by competing with ras-p21 for the effector (GAP) molecule.

To test this hypothesis, Frech et al., (1990) partially purified recombinant rap1A-p21 from E. coli as well as a truncated carboxyl terminal version. These researchers measured the affinity of binding between rap1A-p21 and GAP by the ability of rap1A-p21 to inhibit the effect of GAP on ras-p21 GTPase activity. This binding affinity was tested because it is known that oncogenic mutant forms of ras protein that are not sensitive to GTPase stimulation by GAP still bind to GAP (Vogel et al., 1988). The results showed that GTP-bound rap1A-p21 was a potent inhibitor of GAP-mediated ras-p21 GTPase activity. Inhibition of the interaction between ras-p21 and GAP occurred only when rap1A-p21 was complexed with GTP.

Ohmori et al. (1989) found that in platelets the concentration of rap1A-p21 is approximately 10 times as high as that of ras-p21. This suggests that in vivo, rap1A-p21 may

regulate the interaction between GAP and ras-p21. Furthermore, Krev-1 may suppress transformation of cells by ras oncogenes since GAP is necessary for ras action (Frech et al., 1990).

Based on their results and those of other researchers, Frech et al., (1990) have proposed a model for GAP function. The binding of GAP to ras-p21-GTP may cause a conformational change in ras which is an intermediate in the GTP hydrolysis reaction. GAP itself may undergo a conformational change with binding, and then functions as the effector of ras-p21 action. Hydrolysis of GTP by ras-p21 restores GAP to its original conformation. When GAP binds to rap1A-p21-GTP no conformational changes occur and the activation of GAP hydrolysis does not occur.

Therefore, these recent findings suggest an important role for protease inhibitors such as in the regulation of cellular transformation by ras oncogenes.

B. Intracellular protein metabolism

Studies indicate that there are separate lysosomal and non-lysosomal mechanisms involved in protein breakdown in mammalian cells (Hershko and Ciechanover, 1982). The lysosomal mechanism appears to function in autophagy, especially under conditions of nutritional deprivation.

The non-lysosomal, energy requiring mechanism of protein breakdown was elucidated by studies of an ATP-dependent cell-free proteolytic system from reticulocytes (Hershko et al., 1980). A major component of this system is a small, heat stable, universally conserved protein called ubiquitin. Ubiquitin moieties are covalently linked to target proteins by isopeptide bonds in an ATP-requiring reaction.

The purpose of this section is to review the non-lysosomal mechanism of protein breakdown in mammalian cells as it pertains to the research presented in this thesis.

1. Ubiquitin and ubiquitin-dependent proteolysis

Ubiquitin is a 76 amino acid protein which exists in all eukaryotic cells either free or covalently joined to proteins in the cell nucleus, cytosol or plasma membrane (Monia et al., 1990). It is the most highly conserved and abundant protein in eukaryotes, characteristics suggesting a vital role for the protein. This protein is encoded by a multigene family of natural gene fusions. The family is divided into two classes of polygenes (polyubiquitin) which are composed of direct repeats of the 76 amino acid coding unit. Polyubiquitin genes have been found in yeast (Ozkaynak et al., 1987), Dictyostelium (Müller-Taubenberger et al., 1988), Xenopus (Dworkin-Rastl et al., 1984), chicken (Bond and Schlesinger, 1985), Drosophila (Arribas et al., 1986), trypanosomes (Swindle et al., 1988) and human (Wiborg

et al., 1985). Variations occur in the number of polyubiquitin encoding loci between genomes and in the number of coding repeats in each gene (Monia et al., 1990).

The second class of ubiquitin genes was found to be composed of a single ubiquitin coding unit fused in frame to sequences encoding for unrelated amino acids at the C-terminal side of ubiquitin (Ozkaynak et al., 1987; Müller-Taubenberger et al., 1988; Ohmachi et al., 1989; Lund et al., 1985). Differential regulation of ubiquitin gene expression has been observed in various organisms including Artemia (Sastre et al., 1989).

Ubiquitin has been shown to play a key role in a variety of cellular processes such as protein degradation (Ciechanover et al., 1980., Wilkinson et al., 1980), maintainance of chromatin structure (Matsui et al., 1979., Levinger and Varshavsky, 1982), cell surface receptor function (Siegelman et al., 1986., Yarden et al., 1986) and differential regulation of gene expression (Monia et al., 1990).

The most characterized function of ubiquitin is the ATP-dependent catalysis of proteolysis of proteins. Researchers have estimated that at least 90% of the short-lived proteins within a cell are degraded by the ubiquitin-dependent process (Finely et al., 1984., Gregori et al., 1985). Metabolic instability of a protein is a characteris-

tic that ensures the regulation of its intracellular concentration through changes in the rate of synthesis and degradation.

The ubiquitin-dependent protein degradation process has been divided into two steps for simplicity. One step involves the covalent attachment of ubiquitin to the protein substrate. The second step involves the recognition of the ubiquitin-protein conjugate by a protease which then hydrolyzes the substrate. Covalent conjugation of ubiquitin to other proteins involves the formation of an isopeptide bond between the carboxyl-terminal glycine residue of ubiquitin and the E-amino group of a lysine residue in the acceptor protein (Wilkinson, et al., 1988., Finely and Varshavsky, 1985). The ubiquitin C terminus is activated through the formation of a thiol ester with an activating enzyme (E1) and then transferred to a thiol group on a carrier protein (E2). A ligase called E3 catalyzes the transfer of ubiquitin to substrate protein amino groups. The ubiquitin-protein conjugate is degraded by an ATP-dependent protease and an isopeptidase regenerates ubiquitin.

Researchers have designed various experiments to study selective protein turnover. These include the use of plasmids that encode a specific (modified) proteolytic substrate to test in an <u>in vitro</u> system (Bachmair et al., 1986). Site-directed mutagenesis of both proteolytic substrates and ubiquitin is another approach that has revealed a wealth of information (Chau et al., 1989., Ecker et al., 1989). Site-

directed mutagenesis of ubiquitin has shown that conformational changes in ubiquitin while it is conjugated to a substrate, determine whether the conjugate is stable, de-ubiquitinated or degraded (Ecker et al., 1989). Furthermore, experiments with ubiquitin mutated at its lysine^{48} residue show that the multi-ubiquitin chain in a targeted substrate is necessary for degradation of the protein (Chau et al., 1989).

Experiments to determine the nature of the degradation signal have shown that in a short-lived protein it is the amino-terminal residue of the protein that determines its specificity. This signalling has been termed the N-end rule and is conserved between yeast and mammals (Gonda et al., 1990). Chau et al. (1989) studied modified B-galactosidase proteins with different amino-terminal residues to gain insight into the N-end rule. These researchers found that depending on the nature of the amino-terminal residue, the protein is either short-lived or metabolically stable.

Evidence that the amino terminal residue of a protein is involved in its selectivity by ubiquitin was found by the following observations; (1) proteins that have blocked amino termini by acetylation are not substrates for the ubiquitin system; the same non-acetylated proteins in other species are good substrates; (2) chemical modification of the alpha-amino groups of proteins prevents ubiquitin conjugation; and (3) addition of new amino groups on previously unselected proteins resulted in conjugation to ubiquitin. Therefore,

by the same token, any conformational change in a protein that exposes a previously buried amino-terminal group may render susceptibility to ubiquitin conjugation and protein breakdown (Hershko, 1988).

It has also been found that covalent conjugation of ubiquitin to short-lived proteins is essential for their degradation. Chau et al. (1989) showed that all ubiquitin proteins in a selected, short-lived protein exist as a multiubiquitin chain. One ubiquitin moiety within the chain is linked to an adjacent ubiquitin moiety by an isopeptide bond between the carboxyl-terminal glycine^{76} of ubiquitin and an internal lysine residue in its neighbour. To determine the significance of the multi-ubiquitin chain, site-directed mutagenesis was used to convert the lysine48 residue of ubiquitin to arginine which does not accept ubiquitin. This mutant ubiquitin was expressed in E. coli, purified and added to an ATP-supplemented reticulocyte fraction II containing ^{35}S-labelled arginine-B-galactosidase. The degradation of ^{35}S-arginine-Ꞵ-gal was assayed by measuring acid-soluble radioactivity. Only mono-ubiquitin arginine-Ꞵ-gal was produced after addition of mutant ubiquitin, while wild-type ubiquitin produced mulitiubiquitin derivatives of arginine-Ꞵ-gal. Therefore, mono-ubiquitination of a target protein is not sufficient for its degradation while multi-ubiquitination is necessary.

Chau et al. (1988) suggested that the multi-ubiquitin chain provides binding sites for the ubiquitin-dependent protease which degrades the protein substrate. This hypothesis would explain the necessity of the multi-ubiquitin chain in protein degradation as well as the presence of metabolically stable mono-ubiquitinated intracellular proteins.

However, Varshavsky et al. (1988) suggested that a process of ubiquitination-de-ubiquitination of a protein may conformationally change and restore that protein while its activity remains stable. A ubiquitin moiety could reversibly join to an acceptor protein thereby modulating the acceptor protein (Chau et al., 1988). This assumes that not all ubiquitinated proteins are intermediates in the degradation process and also accounts for the existence of metabolically stable ubiquitin-protein conjugates. It is known that the DNA-associated histones H2A and H2B are not destabilized by ubiquitination <u>in vivo</u> (Finely and Varshavsky, 1985). This type of conjugation would be independent of proteolysis and raises the question of how the cell distinguishes between ubiquitinated proteins destined for degradation. Varshavsky et al. (1988) postulates that ubiquitination of these substrates may result from enzymes that are independent of the N-end rule because they recognize characteristics of the protein other than its amino terminus.

Clearly, many aspects of the ATP-ubiquitin-dependent proteolytic degradation pathway have been elucidated. However, more remains to be determined with respect to the specificity and selectivity of protein turnover. Indeed, the discovery of mono-ubiquitinated metabolically stable long-lived proteins may lead to another form of regulation of protein turnover. This aspect will appear again later in this thesis.

C. The brine shrimp Artemia as a developmental model

The invertebrate Artemia belongs to the phylum Arthropoda and inhabits brine lakes and ponds. The conditions of these ponds are extreme in salinity, almost anaerobic and very basic in pH. Fertilization in Artemia occurs in the oviduct and embryos develop in the ovisac until early gastrulation. At the early gastrula stage two possibilities occur in the fate of the life cycle. One is that the early gastrula remains in the ovisac until it develops into a prenauplius larva and then completes its development through the nauplius larva stage into an adult externally (ovoviviparous reproduction).

The second possibility is that the early gastrula, still in the ovisac, becomes encysted in a tough chitinous shell and enters a dormant state. These dormant cysts are released from the ovisac and are desiccated osmotically and immobilized on land. There is no metabolic activity in the

cysts, however upon rehydration metabolic activity resumes. Following dormancy is a morphogenetic stage in which protein synthesis is initiated from stored mRNP's. Polyribosomes are not present in dormant embryos, but are rapidly formed when development resumes (Golub and Clegg, 1968). The immediate initiation of protein synthesis upon resumption of development suggests that dormant embryos contain mRNA's. Slegers (1989) has shown that functional mRNA's can be isolated from dormant cysts and translated *in vitro*.

RNA synthesis resumes approximately 5 hours after the resumption of development and at nine hours the prenauplii emerge. At approximately 12 hours of development DNA synthesis resumes and then the hatching of prenauplii into nauplii occurs. This starts the period of differentiation and growth which allows the nauplii to become adults.

The advantages of using *Artemia* as an invertebrate developmental model are that relatively synchronous populations can be obtained at various developmental stages. The dormant cysts are abundant and easy to cultivate under controlled conditions and they can be easily released from this state. However, the dormant cysts are impermeable to specific inhibitors and most radio-labelled precursors.

The dormant cysts are resistant to the deleterious effects of anaerobiosis, and previous data showed that they do not utilize trehalose under these conditions in contrast to aerobic cysts which use trehalose for energy metabolism (Ewing and Clegg, 1969). Other researchers suggested that

the nucleotide Gp4G may provide the energy source for maintaining anaerobic cysts (Stocco et al., 1972). To this date very little is known about the type of metabolism sustained by dormant cysts during anoxia. Many researchers in this field are interested in the events that occur during dormancy, and immediately thereafter, when the embryo loses resistance to anoxia after hatching.

The process of development is characterized by a complex series of changes in selective gene expression (Bagshaw, 1980). Therefore these developing embryos are good model systems for the study of gene expression. Furthermore, as an invertebrate developmental system Artemia has provided information on the role of proteases in the invertebrate developmental process.

D. Thiol proteases and thiol protease inhibitors in Artemia embryos

1. Characterization of a cathepsin B-like protease in Artemia embryos

In 1978 studies were initiated to characterize proteases in dormant embryos of the brine shrimp Artemia (Nagainis and Warner, 1979). The initial step in the study was the developement of an assay to detect proteolytic activity resulting from the liberation of amino groups upon hydrolysis of a peptide. Hydrolysis of a peptide bond in a protein

results in the production of two polypeptides with amino termini that react with trinitrobenzenesulfonic acid (TNBS) in alkaline conditions to produce a yellow chromophore which absorbs at 420 nm. Bovine serum albumin (BSA) was used as the substrate for the thiol protease which possesses N-terminal moieties and epsilon groups that also react with TNBS. Therefore, this type of background must be taken into account when recording proteolytic activity.

Subsequent to the development of the TNBS assay, the amount and type of proteolytic activity in Artemia cysts was investigated. In extracts from dormant and prehatched embryos of Artemia, one protease has been discovered that accounts for 90% of the total enzyme activity (Nagainis and Warner, 1979). These researchers found proteolytic activity at pH 4.0 in the postribosomal supernatant from extracts of dormant cysts. When this active fraction was dialyzed, the proteolytic activity at pH 4.0 increased by at least 300%. Varying amounts of the dialyzed protein fraction were assayed for proteolytic activity and it was observed that the initial rate of proteolysis was low.

After anion exchange chromatography on a DEAE-cellulose column, the proteolytic activity eluted as a single peak. These fractions were assayed and did not show the initial lag in proteolytic activity. Therefore, the inhibitory substance had been removed by anion-exchange chromatography. However, when fractions containing enzyme activity were pooled and assayed with aliquots from other column frac-

tions, the lag in activity was restored. Therefore, it was understood that Artemia cysts contain a non-dialyzable protease inhibitor as well as a dialyzable inhibitory factor.

The protease has since been purified to homogeneity and characterized from the cytosol (Warner and Shridhar, 1980, 1985; Warner, 1987; Perona and Vallejo, 1985; Nagainis and Warner, 1979) and particulate fractions of dormant cysts (Perona and Vallejo, 1982). Studies revealed that the enzyme is a cathepsin B-like protease and catalyzes the hydrolysis of BANA and N-Cbz-Arg-Arg-NA which are well known mammalian cathepsin B substrates. The cytosol protease is sensitive to thiol protease blocking agents such as leupeptin, antipain, chymostatin, Ep-475, iodoacetate, Cu, and Hg, but is not sensitive to pCMB and NEM (Warner and Shridhar, 1980, 1985). The protease is uneffected by pepstatin, ovomucoid, STI and PMSF (Warner, 1987).

The cathepsin B-like protease was found to be a glycoprotein composed of two subunits of 31.5 and 25.9 kDa (Warner and Shridhar, 1985). The protease focuses at pH 4.6 and 5.1 on isoelectric focusing gels. This enzyme degrades a variety of substrates including protamine sulfate, BSA, Artemia lipovitellin, protein synthesis elongation factor 2 (EF-2) and hemoglobin in the pH range of 3.5 to 4.5.

A major developmental event that occurs in Artemia following resumption of development is yolk platelet utilization. Yolk platelets are the major storage material of the egg and are composed of vitellogenin. Vitellogenin is con-

verted into lipovitellin in the oocyte. By the first 24 hours in Artemia development, 50% of the yolk platelets have been utilized (Sillero et al., 1980) and degradation of lipovitellin occurs simultaneously. Hydrolysis of lipovitellin (LV-alpha-1, 190 kDa) in vivo results in the sequential production of polypeptides designated as alpha-1 to alpha-10, beta, gamma and epsilon according to their migration positions in sodium dodecyl sulfate polyacrylamide gels (De Chaffoy de Corcelles et al., 1980).

Warner and Shridhar (1985) investigated the hydrolysis of lipovitellin by the cyst cathepsin B-like protease at pH 6.0 to 6.5. They found that the pattern of lipovitellin hydrolysis by the thiol protease in vitro paralleled that in vivo in Artemia. This correlation of in vitro lipovitellin degradation by the cyst thiol protease with the in vivo degradation pattern suggests a role for the protease in yolk metabolism.

Further studies of extracts from dormant Artemia embryos revealed a depressed translational capacity in cell free translational systems (Yablonka-Reuveni and Warner, 1979). These researchers found the presence of low molecular weight polypeptides in Artemia extracts that accepted ADP-ribose. The fragments were then shown to be specific degradation products of native EF-2 and that the enzyme producing them may be present in a sequestered form in crude extracts of dormant cysts.

At pH 6.0, most Artemia cytosol proteins are resistant to hydrolysis by the cathepsin B-like protease, while the enzyme has a high degree of specificity for Artemia EF-2. Studies showed that the cathepsin B-like protease selectively hydrolyzes the 95 kDa EF-2 into lower molecular weight fragments of 54, 45, 40, and 38 kDa at pH 6.0-6.5, and these fragments appear to be the same as those found in embryo extracts after hatching (Warner and Shridhar, 1985). The non-dialyzable inhibitor is resistant to hydrolysis by the thiol protease in the pH range of 6.0-6.5 and therefore it has the capacity to regulate its activity.

In Artemia embryos subjected to anaerobic conditions, the intracellular pH decreases to approximately 6.3. Protein synthesis is depressed under these conditions and EF-2 is inactivated (Warner and Shridhar, 1985). The thiol protease is active on EF-2 under these conditions in vitro, and therefore may be activated in vivo to shut down protein synthesis. Furthermore, in this case regulation of the thiol protease activity is vital to prevent inappropriate proteolysis. Barnaerts has observed a 10-fold increase in thiol protease activity in the postribosomal fraction of hydrated dormant Artemia cysts 90 minutes after exposure of the embryo to anaerobic conditions (Warner and Shridhar, 1985). Since the protein synthetic machinery is not operational in dormant embryos, the increase in protease

activity may be due to a shift in equilibrium between free and inhibited forms of the enzyme (Warner and Shridhar, 1985).

Various researchers have attempted to determine whether developmental changes in thiol protease activity in Artemia correlate with synchronous changes in its respective substrates (Marco et al., 1980; Perona and Vallejo, 1981; Warner and Shridhar, 1980, 1985). However, the literature on this subject contains conflicting results. The problems are due mainly to the use of sodium hypochlorite for sterilizing cysts. Between 0 and 16 hours development of hypochlorite-sterilized cysts, protease activity is extremely low in the cytosol. However, cytosol fractions from nauplii (20-36 hours) are not affected. It has been found that the inactivation and irreversible dissociation of ribosomes in dormant cysts occurs when associated with oxidants such as sodium hypochlorite, hydrogen peroxide and cationic detergents (Warner and Shridhar, 1985).

Another factor that contributes to discrepencies in levels of thiol protease activity throughout development is its association with low molecular weight inhibitors. If inhibitors are not separated from their respective enzymes prior to the assay, an underestimation of enzyme activity may result.

The developmental profile of cathepsin B-like activity was determined avoiding the use of sodium hypochlorite (Warner and Shridhar, 1985). The results showed that the

thiol protease activity was highest in extracts from dormant cysts and decreases gradually during development until after hatching and then the total protease level remains constant for an additional 20 hours. Total thiol protease activity represents both enzyme free of inhibitors and that bound to inhibitors. These researchers determined that approximately 40% of solubilized thiol protease from dormant Artemia embryos is inactive. At the time of emergence of the prenauplius larvae, the distribution of free and bound thiol protease drastically changes. The thiol protease activity represented by the free form increases while that of the bound decreases. This observation suggests that in pre-emerged larvae, the thiol protease may be regulated by thiol protease inhibitors.

2. Characterization of thiol protease inhibitors in dormant Artemia embryos

As mentioned in the above section, extracts from dormant Artemia embryos contain a dialyzable as well as a non-dialyzable inhibitor (Nagainis and Warner, 1979). The non-dialyzable inhibitor is present in the postribosomal fraction of such extracts and can be separated from the thiol protease by chromatography on DEAE-cellulose columns. This inhibitor was partially purified by ion-exchange and gel filtration chromatography (Warner, 1987, 1989).

Chromatography of the inhibitor preparation on a column of CM-cellulose resulted in the fractionation of two proteins possessing thiol protease inhibitor (TPI) activity. Both inhibitors were found to have molecular weights of approximately 9.0 kDa by gel filtration and 1-2 ug/ml of inhibitor preparation inhibited the cathepsin B-like thiol protease activity by 50% (ID_{50}) at pH 5.0 and 40 C (Warner, 1987). When both inhibitors were incubated with the cathepsin B-like protease at pH 4.0 and 40 C the inhibitor was inactivated. The inhibitors are effective on the protease in a pH range of 5.5-6.5 and specifically at pH 6.0-6.5 when EF-2 is the substrate.

Measurements of thiol protease inhibitor activity in protease-free extracts from Artemia embryos and larvae show a significant increase in inhibitor activity in embryos during the first 6-9 hours. Over the remaining 27-30 hours of Artemia development, the TPI activity rapidly decreases (Warner, 1987).

The ratio of thiol protease concentration to inhibitor concentration can be calculated using specific activity data for the enzyme and inhibitor at different stages of development. Results from this type of analysis show that after hatching of prenauplius larvae (9 hours), the enzyme to inhibitor ratio is at least 16 compared to 1 in dormant cysts. This suggests a reduction in control of thiol protease activity by the thiol protease inhibitor. This is in

accordance with previous observations on the degradation of EF-2 and decreased protein synthesis capacity of extracts from nauplii (Yablonka-Reuveni and Warner, 1979).

More recent studies using a different purification procedure suggest that there are more than two low molecular weight thiol protease inhibitors in extracts from dormant Artemia cysts (Warner, 1989). These results were obtained from inhibitor preparations that had been subjected to DEAE-cellulose chromatography, gel filtration on a column of Sephadex G-75 (superfine) and fast protein liquid chromatography (FPLC) on a cation exchange column. FPLC revealed thiol protease inhibitor activity in at least four protein peaks which had not been completely resolved and each peak was heterogeneous as determined by SDS-PAGE.

Several fractions from the cation exchange column (FPLC) were studied for pH stability and kinetics of inhibition of the cathepsin B-like protease (Warner, 1987). These studies revealed that the thiol protease inhibitors are stable at neutral and alkaline pH but are inactive at acidic pH's.

Previous kinetic studies on Sephadex G-75 purified inhibitor preparations revealed that the extent of inhibition of the thiol protease is pH-dependent. The pH optimum of the thiol protease is 3.6-3.8 (Warner, 1987) while the inhibitor(s) is(are) most active at pH 5.0 and 6.0. The inhibitor(s) was (were) found to be non-competitive with a Ki value of 1.7×10^{-11}M.

Therefore, at this stage the Artemia inhibitors resemble mammalian low molecular weight thiol protease inhibitors in their molecular weight and mechanism of inhibition. However, they do not exhibit stability to extremes in temperature and pH as do most mammalian low molecular weight intracellular TPI's (Lenney, 1980; Kominami et al., 1982).

Control of protease activity in Artemia embryos may be influenced by the ionic environment, intracellular pH, compartmentalization and the presence of protease inhibitors (Warner, 1987). In Artemia embryos, the pH during aerobic development is at least 7.9 (Busa et al., 1982). Since the pH optimum of the thiol protease in vitro is 3.5-3.8, it must be stabilized in Artemia embryos by some mechanism. The thiol protease may be protected in lysosomes as suggested by Perona and Vallejo (1982) which is typical of thiol proteases in most mammalian systems (Katunuma and Kominami, 1985). Alternatively or simultaneously, the thiol protease may be protected by the low molecular weight thiol protease inhibitors present in Artemia embryos. This is in accordance with previous results showing that 40% of the thiol protease activity in embryo extracts is in a bound form (Warner and Shridhar, 1980).

As mentioned previously, the thiol protease inhibitors may be involved in the regulation of protein synthesis, in yolk metabolism and during anoxia, indirectly through the regulation of thiol protease activity in Artemia embryos. Both the thiol protease and its inhibitor(s) should be lo-

calized in Artemia embryos by immunochemical techniques. Moreover, to obtain a better understanding of the involvement of proteases and their inhibitors in the development of Artemia embryos, the low molecular weight thiol protease inhibitors should be purified and fully characterized.

II. MATERIALS AND METHODS

1. Sources

The *Artemia* cysts used in all experiments were from the Great Salt Lakes in Utah (lot number 12715, Sanders Brine Shrimp Company). DEAE-Sephadex, Sephadexes G-25, G-50, and G-75 and the Mono S cation exchange column were from Pharmacia. Protamine sulfate was from Calbiochem and 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) was from ICN Biomedicals. Ubiquitin (from bovine red blood cells and Baker's yeast) and the low molecular weight standards (mw-SDS-17) were from Sigma Chemical Company (St. Louis, MO). Ultrafiltration membranes and centricon filters were from Amicon (Boston, MA). The high molecular weight standards and protein stains were from Bio-Rad Laboratories (Richmond, CA., U.S.A.). All other chemicals were of reagent grade.

2. Thiol protease and thiol protease inhibitor assays

The determination of *Artemia* cyst thiol protease inhibitor activity during the purification procedure was carried out using a partially purified thiol protease preparation (DEAE-Sephadex fraction) as described previously (Warner and Shridhar, 1985) with protamine sulfate as the substrate. The activity of the enzyme was based on the amount of amino groups liberated from protamine sulfate by the protease

(TNBS assay) in the presence of column fractions according to the procedure of Nagainis and Warner (1979). Each reaction vessel contained 0.1M sodium acetate, pH 5.0, 1mM EDTA, 1mM DTT, 7.5% glycerol, 4 mg/ml protamine sulfate, 10-14 milliunits of Artemia thiol protease and column fractions to be tested for inhibitor activity in 200 ul total volume. Aliquots of 50 ul were removed at the desired times (0 to 30 min.) and the extent of amino group liberation (TNBS assay) was determined compared to control vessels containing buffer instead of the column fractions.

One unit of thiol protease inhibitor activity (IU) was defined as that amount of protein which inhibits one unit of thiol protease activity (EU), where one EU equals the amount of enzyme liberating 1 µmol/min of amino groups using l-arginine-HCl as the standard. On occassion, inhibitor activity was also expressed as percent inhibition (%I). For the kinetic studies similar reaction vessels were set up except that 1-2 mU of pure Artemia thiol protease was used. The enzyme had been purified by A. H. Warner as previously described (Warner and Shridhar, 1985).

3. Preparation of cysts for inhibitor isolation

To prepare Artemia cysts for homogenization, 50 grams dry weight were hydrated on ice overnight in 0.25 M NaCl. The hydrated cysts were washed three times in ice-cold water during which time floating cysts and debris were removed by

suction. The remaining fully hydrated cysts were collected in a glass filter by suction. This process routinely yielded 1.5 g hydrated cysts (wet weight) per gram dry cyst.

4. Isolation of thiol protease inhibitors from Artemia cysts

Hydrated cysts were homogenized for 15 minutes at 4C in 5 volumes of 50 mM Tris-HCl, pH 7.3 containing 5 mM KCl, 1 mM DTT and 10 mM $MgCl_2$ using a motorized mortar and pestle. The homogenate was centrifuged at 10,400 x g for 30 minutes and the supernatant filtered through a cheesecloth-glass-wool-cheescloth sandwich to remove any floating material. This filtrate was centrifuged in an ultracentrifuge (Beckman L5-65) at 150,000 x g for 150 minutes to obtain the postribosomal supernatant (PRS). The PRS was adjusted to 25% ammonium sulfate and the insoluble protein allowed to form overnight in an ice bath. The protein insoluble in 25% ammonium sulfate was removed by centrifugation at 4080 x g for 30 minutes and the soluble material filtered through another cheesecloth-glasswool sandwich. The filtrate was adjusted to 75% ammonium sulfate and the protein allowed to precipitate for 4-5 hours in an ice bath. The protein which precipitates between 25-75% ammonium sulfate was collected by centrifu- gation at 10,400 x g for 60 minutes and stored at $-10^{o}C$ until further use.

5. Purification of Artemia thiol protease inhibitors

The 25-75% ammonium sulfate precipitates were suspended in 15 mM potassium phosphate, pH 6.8 containing 25 mM KCl and desalted on a column of Sephadex G-25 (4 x 98 cm) equilibrated with the same buffer. The orange-coloured material which eluted in the void volume was collected and subjected to ion exchange chromatography on DEAE-Sephadex (A-50) to separate the inhibitor from the thiol protease and other acidic proteins in the preparation. The protease inhibitor appeared in the flow through material and wash of the DEAE-Sephadex column (between 200 and 600 ml). It was concentrated by ammonium sulfate precipitation (75%) overnight in an ice bath. The crude inhibitor protein/precipitate was collected by centrifugation at 16,300 x g for 30 minutes. The protein was resuspended in 15 mM potassium phosphate, pH 6.8 containing 25 mM potassium chloride and 10% glycerol and concentrated by ultrafiltration to 1-2 ml on a Diaflo YM-2 membrane.

To further purify the inhibitor, the YM-2 concentrate was subjected to chromatography on a column (2.5 x 110 cm) of Sephadex G-75 (superfine) equilibrated with 15 mM potassium phosphate, pH 6.8 containing 25 mM potassium chloride and 10% glycerol. Each column fraction was assayed for UV-absorbing material (protein) at 280 nm and for thiol protease inhibitor activity as described above. The column

fractions containing protease inhibitor activity were pooled and concentrated by ultrafiltration through a Diaflo YM-2 membrane.

The concentrated protease inhibitor (from Sephadex G-75) was applied to a column (2.0 x 53.6 cm) of Sephadex G-50 equilibrated with the same buffer as used in the previous step. Again column fractions were assayed for protein at 280 nm and for protease inhibitor activity. Column fractions containing inhibitor activity were pooled and concentrated by ultrafiltration through a YM-2 membrane. This chromatographic step on Sephadex G-50 was performed up to four times, each time recycling fractions containing the least amount of protein with the highest activity.

6. Chromatography on cation exchange columns

a. Fast protein liquid chromatography (FPLC)

The thiol protease inhibitor preparation from the final Se- phadex G-50 column was concentrated and subjected to fast protein liquid chromatography on a Mono S cation exchange column (0.5 x 5.0 cm). Prior to FPLC the sample and column were equilibrated with 20 mM sodium acetate, pH 5.0 containing 1 mM EDTA and 10% glycerol. Six minutes (or ml) after the sample was injected, a linear gradient of 300 mM NaCl was started to elute the protein over a 30 minute period at 1ml/min. The column effluent was monitored at 280

nm using a Gilson Holochrome detector and selected column fractions were assayed for inhibitor activity. The active fractions were pooled and concentrated using Centricon-3 microconcentrators.

b. Chromatography on CM-cellulose

In one experiment, the thiol protease inhibitor preparation from the final Sephadex G-50 column was concentrated and subjected to cation exchange chromatography on a carboxymethyl (CM)-cellulose column (1 x 27 cm). Prior to chromatography this column was equilibrated in 20 mM sodium acetate, pH 5.0 containing 10% glycerol and the inhibitor preparation was equilibrated in the same buffer on a small Sephadex G-25 column.

The sample was washed on the column with the above buffer and the proteins were eluted with 20 mM sodium acetate, pH 5.0 containing 10% glycerol and 50 mM KCl. After 150 ml had washed through the column, any protein still on the column was eluted with a linear gradient of 500 mM KCl. Fractions from the column were collected in 150 ul of 4 parts 0.5 M sodium acetate (pH 8.5) and 1 part (0.1 M) sodium hydroxide in order to raise the pH from 5.0 at which the inhibitors are not stable.

7. High performance liquid chromatograpy (HPLC)

Each protease inhibitor fraction from the Mono S or CM-cellulose column (after concentration) was analyzed on a C-18 reversed phase column (4.6 x 250 mm Chemopack) driven by a Gilson HPLC system. The column was equilibrated with 12% acetonitrile containing 0.1% trifluoroacetic acid (TFA) immediately before sample injection. After the sample was injected the protein was eluted at a flow rate of 1 ml/min. with a linear gradient of acetonitrile to either 36%, 45% or 60% with 0.1% TFA over 40 minutes. The column effluent was monitored at 214 nm using a Gilson Holochrome detector. Each fraction was analyzed for protease inhibitor activity as described above following renaturation of the proteins eluting from the HPLC column as described below.

8. Renaturation procedure for HPLC purified proteins

The renaturation of TPI protein from HPLC analyses was performed essentially as described by Hager and Burgess (1980). Individual column fractions were lyophilized to dryness, suspended in 4 µl of 6 M guanidine-HCl and incubated at room temperature for four hours. Then 196 µl of renaturation buffer (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 20% glycerol, and 0.1 M KCl) was added and the mixture was allowed to incubate overnight at 4°C. Control vessels containing only 4 µl of 6 M guanidine-HCl

and 196 µl of renaturation buffer were also set up to use in control reactions. The presence of protease inhibitor activity in each of the renatured fractions was tested as described above.

9. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out using 7-18% gradients of polyacrylamide minigels containing 0.1% SDS according to the procedure of Laemmli (1970). Protein was stained using the Bio-Rad silver reagent after fixation of the gel in 40% methanol/10% acetic acid (v/v) followed by 10% ethanol/5% acetic acid (v/v). The molecular weight standards were: phosphorylase b (94 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.2 kDa).

10. SDS-urea polyacrylamide gel electrophoresis

The purity and molecular weights of the various Artemia thiol protease inhibitors were analyzed on 15% acrylamide gels containing 0.1 M sodium phosphate, pH 7.2, 0.1% SDS, and 6 M urea according to the procedure of BRL (1984). Pro-

tein was stained using the Bio-Rad silver reagent after fixation of the gel in 40% methanol/10% acetic acid (v/v) then 10% ethanol/5% acetic acid (v/v).

The molecular weight of the Artemia thiol protease inhibitors was determined using the following molecular weight markers, all of which were fragments of myoglobin: myoglobin backbone (17 kDa), fragments I and II linked together (14.4 kDa), fragment I (8.2 kDa), fragment II (6.2 kDa) and fragment III (2.5 kDa).

11. Cyanogen bromide digestion of purified Artemia proteins

The most abundant protein with inhibitor activity from the Mono S column was subjected to reverse phase chromatography on a C-18 column and the eluted protein lyophilized. This sample was dissolved in 44 µl of 70% trifluoroacetic acid (TFA) and then 6.3 µl of 50 mg/ml cyanogen bromide (CNBr) was added. Control samples containing only 44 µl of 70% TFA and 6.3 µl of 50 mg/ml CNBr were also prepared. These preparations were incubated at room temperature for 48 hours and then diluted with 450 µl of water. The mixtures were then frozen, lyophilized to dryness and resuspended in an additional 450 µl of water. After repeated lyophylization the contents of each reaction vessel were suspended in 100 µl of 12% acetonitrile with 0.1% TFA and chromatographed on a C-18 reverse phase column to collect CNBr generated fragments.

12. Amino acid sequence analysis

The amino acid sequence of the CNBr fragments was determined by automatic Edman degradation at the University of Toronto under the direction of Dr. Max Blum.

13. Kinetic analysis of the *Artemia* thiol protease inhibitors

The mechanism of inhibition of the *Artemia* thiol protease by each inhibitor preparation was determined in reaction mixtures containing varying amounts of inhibitor and substrate (protamine sulfate) with a constant amount of purified *Artemia* thiol protease (0.6 µg or 48 mU) in a buffer containing 0.1 M sodium acetate, pH 5.0, 10% glycerol, 1 mM EDTA and 1 mM DTT. Inhibitor units were measured as an indication of enzyme velocity in the presence of different amounts of inhibitor and a double reciprocal plot was constructed from the data.

The temperature of the reaction was maintained at 30°C and aliquots were taken at 0, 10 and 15 minutes incubation. An equation for each line was generated by a linear regression analysis of the data points. In calculations of kinetic constants, a molecular weight of 7.5 kDa was used for protamine sulfate and an average of 12.3 kDa for the inhibitor protein.

14. Protein determination

The protein content of selected samples was determined by the method of Bradford (1976) with bovine serum albumin as standard. Protein content of column eluates was determined by measuring the absorbance at 280 nm.

III. RESULTS

A. Purification of multiple thiol protease inhibitors from dormant Artemia embryos

A purification protocol was developed for the isolation and purification of multiple low molecular weight thiol protease inhibitors from dormant Artemia embryos based on procedures previously initiated by Warner (1989).

To prepare Artemia cysts for homogenization, 50 g dry weight were hydrated overnight in 0.25 M sodium chloride on ice. The hydrated cysts were washed and floating cysts and debris were removed by suction. This process routinely yielded an average wet weight of 145 g.

The hydrated, washed cysts were homogenized at 4°C in five volumes of 50 mM Tris-HCl, pH 7.3, containing 5 mM KCl, 1 mM DTT and 10 mM $MgCl_2$. The homogenate was centrifuged at 10,400 x g for thirty minutes and the supernatant was filtered to remove floating materials. This filtrate was centrifuged at 150,000 x g for 150 minutes to obtain the postribosomal supernatent which was adjusted to 25% ammonium sulfate. The protein insoluble in 25% ammonium sulfate was removed by centrifugation at 4,080 x g for thirty minutes and the soluble material was filtered again to remove additional floating material. The filtrate was adjusted to 75% ammonium sulfate and the protein allowed to precipitate

for 4-5 hours. The protein that precipitated between 25-75% ammonium sulfate was collected by centrifugation at 10,400 x g for sixty minutes.

The 25-75% ammonium sulfate precipitate was resuspended in 15 mM potassium phosphate, pH 6.8, and 25 mM potassium chloride and desalted on a Sephadex G-25 column (superfine, 4 x 98 cm).

This preparation was subjected to anion exchange chromatography on DEAE-Sephadex to separate the protease inhibitor(s) from the thiol protease(s). The protein which did not bind to the DEAE-Sephadex contained most of the thiol protease inhibitor (TPI) activity. The column effluent was concentrated by ammonium sulfate precipitation (75%), and after centrifugation at 4,080 x g for thirty minutes to collect the precipitate, it was resuspended in 15 mM potassium phosphate, pH 6.8, containing potassium chloride and 10% glycerol and concentrated to 10 mL on a Diaflo YM-2 membrane.

This concentrated material was applied to a Sephadex G-75 column (superfine, 2.5 x 93 cm) equilibrated in the above buffer. The material which eluted with this buffer was assayed for protein at 280 nm and for TPI activity using a partially purified thiol protease preparation and protamine sulfate as substrate at pH 5.0 (see Materials and Methods, TNBS assay). The column fractions containing inhibitor activity were pooled and concentrated by ultrafiltration using a Diaflo YM-2 membrane (Amicon).

The results of Sephadex G-75 chromatography are shown in Figure 1, panel A. These results indicated that the elution position of the thiol protease inhibitor activity from the column is very broad and in the low molecular weight region.

The protein fraction from the Sephadex G-75 column with TPI activity was concentrated on a YM-2 filter, then applied to a Sephadex G-50 column (superfine, 2.0 x 41 cm) equilibrated with the same buffer as above (see Figure 1B). The protein and activity profiles were determined as described for the Sephadex G-75 gel filtration step. The column fractions containing inhibitor activity were pooled and concentrated by ultrafiltration on a Diaflo YM-2 membrane (Amicon). The thiol protease inhibitor preparation was rechromatographed on a second Sephadex G-50 column (see Figure 1C) and the fractions containing inhibitor activity were pooled and concentrated as above.

From the results in Figure 1 (panels A, B and C) it was clear that the thiol protease inhibitor exhibited a broad elution volume from the gel filtration column and was of low molecular weight. Moreover, the results in Figure 1, panel C, show that the TPI activity may be composed of two fractions. This observation was noticed constantly on all second Sephadex G-50 column analyses.

In several experiments, four successive Sephadex G-50 (superfine) gel filtration steps were performed, but they were found not to alter significantly the composition or

Figure 1. Purification of thiol protease inhibitor preparations by gel filtration chromatography.

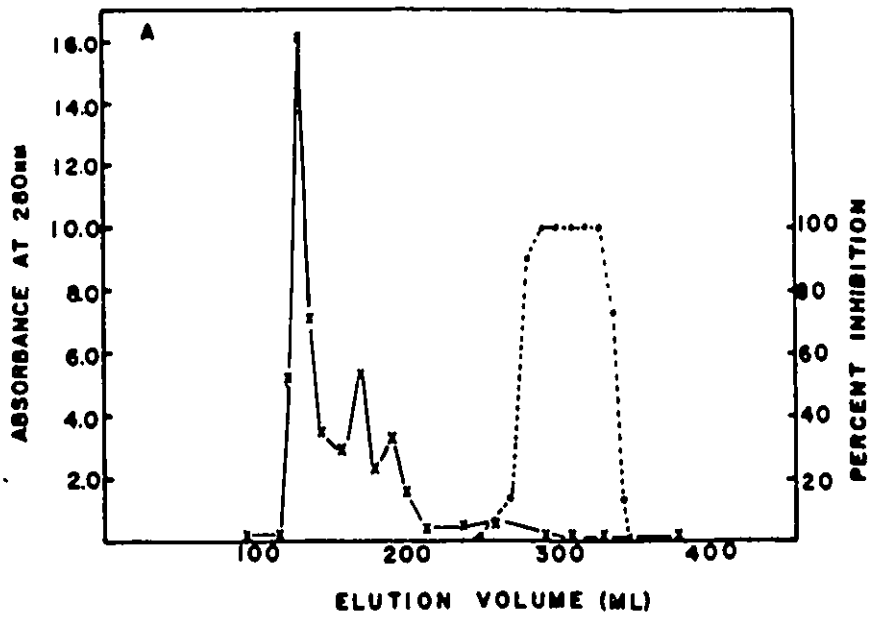
Panel A: Elution of TPI activity from a Sephadex G-75 (superfine) column (2.5 x 93 cm). The starting material was the DEAE-Sephadex treated inhibitor preparation (effluent).

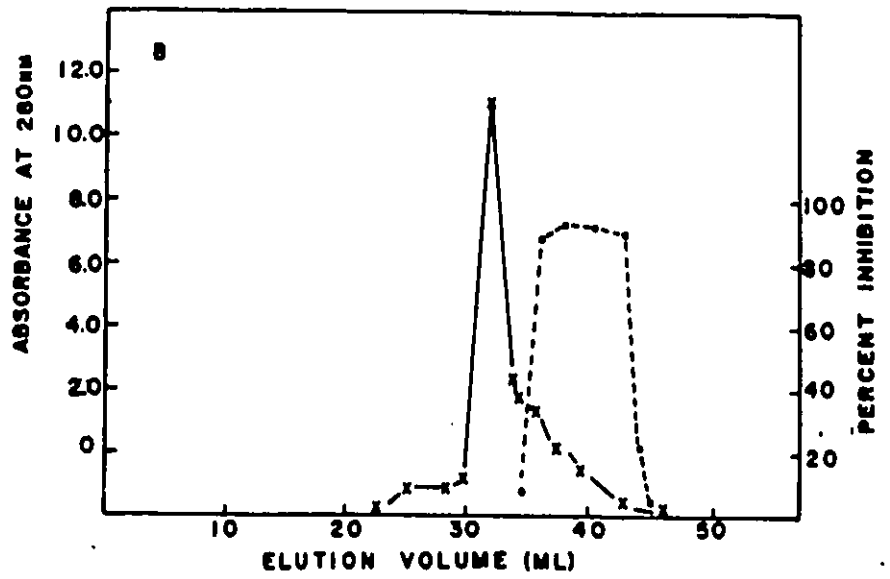
Panel B: Elution of TPI activity from Sephadex G-50 (superfine) column (2.0 x 41 cm). The starting material was pooled and concentrated from Sephadex G-75 shown in panel A.

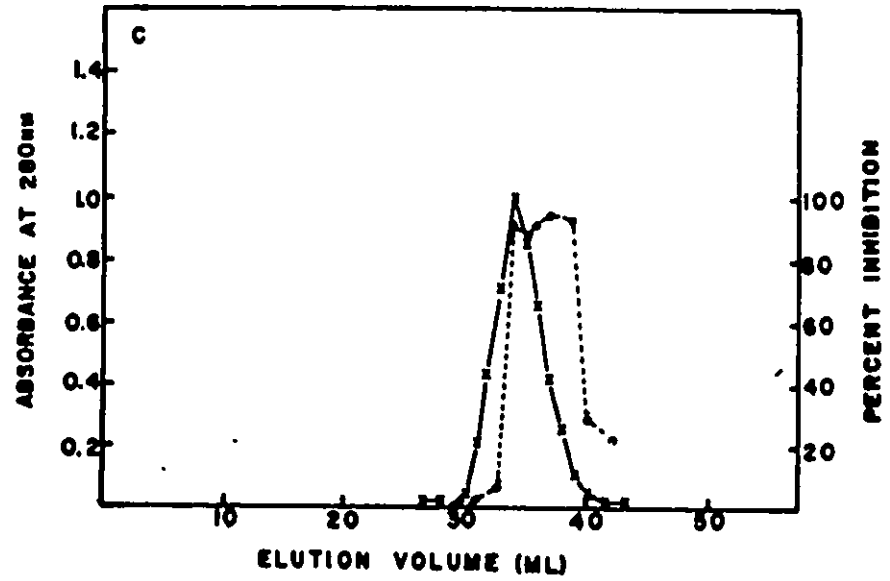
Panel C: Elution of TPI activity from a second Sephadex G-50 (superfine) column (2.0 x 39.5 cm). The starting material was from the first Sephadex G-50 column shown in panel B.

●---------●, inhibitor activity (as percent inhibition)

x---------x, protein as measured by absorbance at 280 nm.


A
ABSORBANCE AT 280nm
PERCENT INHIBITION
ELUTION VOLUME (ML)


B
ABSORBANCE AT 280nm
PERCENT INHIBITION
ELUTION VOLUME (ML)


C
ABSORBANCE AT 280nm
PERCENT INHIBITION
ELUTION VOLUME (ML)

specific activity of the thiol protease inhibitor preparation. Therefore, results are included in which only two Sephadex G-50 steps were performed.

The protein content and inhibitor activity after each of the above steps in the gel filtration process are summarized in Table 1. Aliquots from each step in the purification process were also analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The results of these experiments are shown in Figure 2 and show the enrichment of low molecular weight proteins in the range of 5.2 to 12.0 kDa (see arrows). The second Sephadex G-50 gel filtration step (lane 7) significantly enriched these low molecular weight proteins as observed by SDS-PAGE and also increased the specific activity of thiol protease inhibitor preparation (see Table 1).

Table 1. Summary of steps in the purification of thiol protease inhibitor preparations from *Artemia* dormant cysts (a).

Fraction	Protein (mg)	Specific inhibitor activity (mIU/mg)	Total inhibitor activity (mIU)	% of original activity
25-75% $(NH_4)_2SO_4$	1987	n.d.	n.d.	100
DEAE-Sephadex (b)	473	473	223,729	100
G-75 Sephadex	18.6	3946	73,553	32.8
1st G-50 Sephadex	8.7	5565	48,471	21.6
2nd G-50 Sephadex	4.2	8856	37,372	16.7

(a) represents protein from a 100 g dry cysts as starting material.

(b) effluent

Figure 2. Analysis of Artemia proteins in the TPI fraction at different stages of purification by SDS-polyacrylamide gel electrophoresis.

Samples of selected TPI preparations shown in Table 1 were electrophoresed on a 7-18% polyacrylamide gel in the presence of 0.1% SDS.

Lanes 1 and 8: standard molecular weight proteins;
phosphorylase b (94 kDa),
bovine serum albumin (66.2 kDa),
ovalbumin (42.7 kDa),
carbonic anhydrase (31.0 kDa),
soybean trypsin inhibitor (21.5 kDa),
lysozyme (14.4 kDa), and
aprotinin (6.2 kDa).

Lane 2: 26 µg of the post-ribosomal fraction.

Lane 3: 28 µg of the 25-75% ammonium sulfate precipitate (after Sephadex G-25).

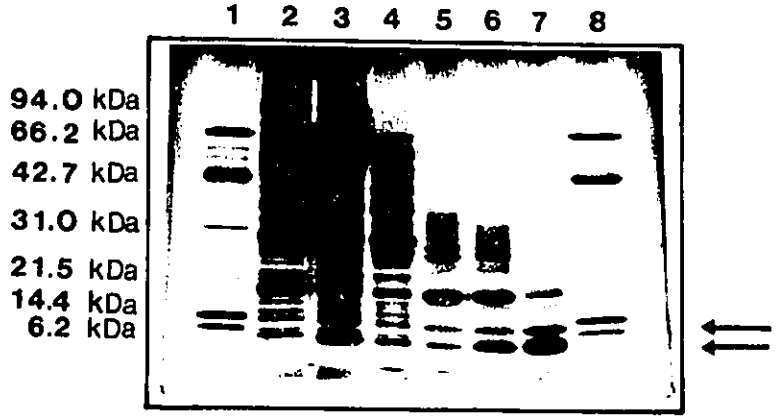
Lane 4: 18 µg of the DEAE-Sephadex effluent.

Lane 5: 7.4 µg of the Sephadex G-75 fraction.

Lane 6: 5.9 µg of the first Sephadex G-50 fraction.

Lane 7: 6.0 µg of the second Sephadex G-50 fraction.

The arrows represent enriched fractions.


1 2 3 4 5 6 7 8
94.0 kDa
66.2 kDa
42.7 kDa
31.0 kDa
21.5 kDa
14.4 kDa
6.2 kDa

B. Cation exchange chromatography of thiol protease inhibitor preparation

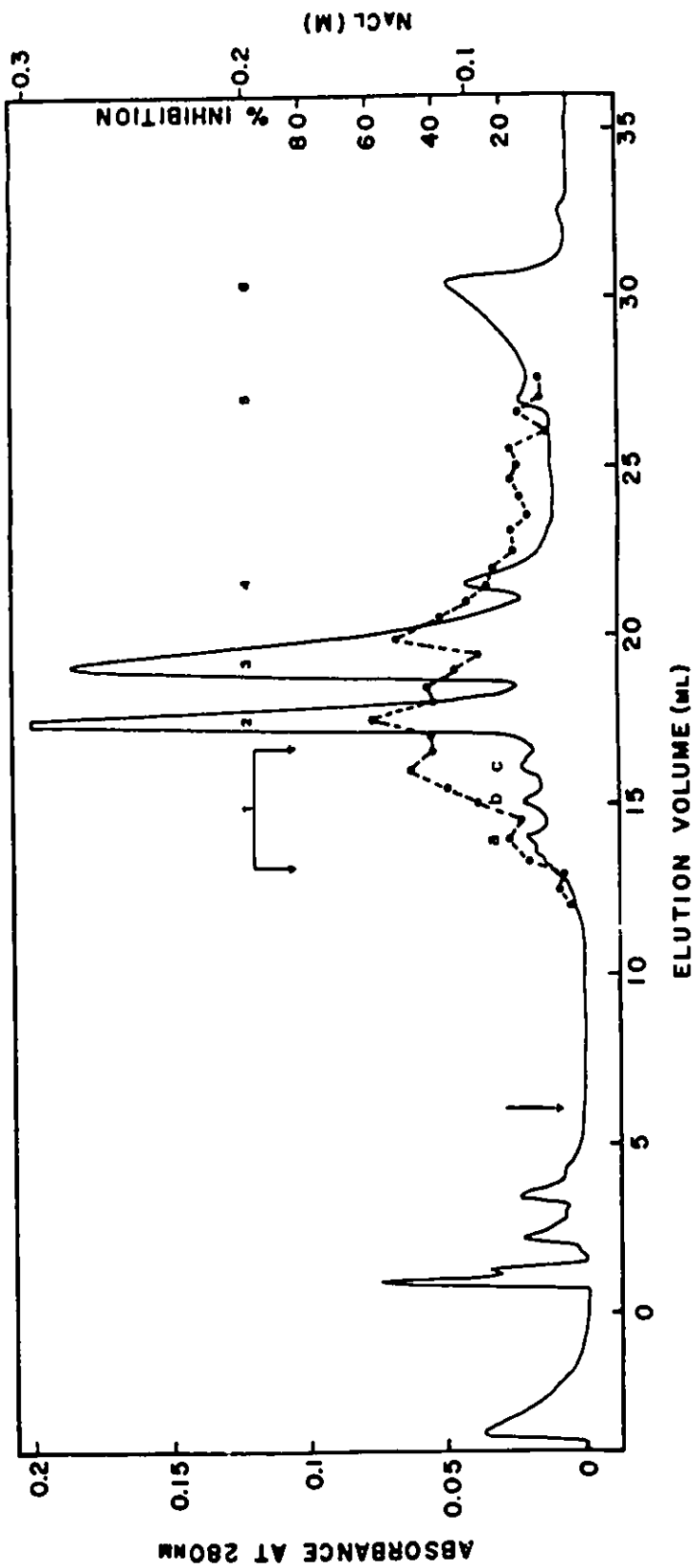
To further purify the thiol protease inhibitor(s), aliquots of Sephadex G-50 preparations were subjected to fast protein liquid chromatography (FPLC) on a Mono S cation exchange column (0.5 x 5.0 cm). The column was equilibrated with 20 mM sodium acetate, pH 5.0, containing 1 mM EDTA and 10% glycerol. Fractions from the Mono S column were monitored at 280 nm and collected in 20 µl of 0.25 mM sodium hydroxide to neutralize the column fraction (the inhibitors were previously found to be unstable below pH 6.0). Each fraction was assayed for thiol protease inhibitor activity, and the active fractions were pooled and concentrated using Centricon-3 microconcentrators. During the concentration process, the buffer was changed to 15 mM potassium phosphate, pH 6.8, containing 25 mM potassium chloride and 10% glycerol.

A typical protein and activity profile resulting from FPLC of a Sephadex G-50 purified thiol protease inhibitor preparation is illustrated in Figure 3. These results revealed a broad range of thiol protease inhibitor activity which required subsequent analysis. Each of fraction 1 through 6 was rechromatographed separately on the Mono S column, and assayed for thiol protease inhibitor activity.

Figure 3. Semi-preparative fast protein liquid chromatography of an Artemia cyst TPI preparation.

An aliquot (2 mg) from the concentrated Sephadex G-50 inhibitor preparation was equilibrated with the starting buffer and applied to a Mono S cation exchange column (0.5 x 5.0 cm), previously equilibrated with 20 mM sodium acetate, pH 5.0, containing 1 mM EDTA and 10% glycerol. After a 6 ml wash in starting buffer, the protein was eluted with a linear gradient (30 min.) of NaCl (to 0.3 M) in starting buffer at 1 ml/min. Protein was monitored at 280 nm and each fraction collected (0.5 ml) was assayed for TPI activity using the TNBS assay. The solid line represents the actual tracing from the recorder and the arrow indicates the beginning of the 30 minute linear gradient. Fractions pooled for further analysis are indicated by the numbers.

●----------●, inhibitor activity (as percent inhibition).


NACL (M)
0.3
0.2
0.1
% INHIBITION
80
60
40
20
35
30
25
20
15
10
5
0
ELUTION VOLUME (ML)
0.2
0.15
0.1
0.05
0
ABSORBANCE AT 280NM
1
2
3
4
a
b
c

Only the results from the analyses of fractions 1 and 2 are included in the results because they contained the most significant amount of TPI activity.

Each of the protein peaks in fraction 1 from the Mono S column was pooled separately into three separate fractions (see Figure 3) and rechromatographed on the same column. The results of the rechromatography of fractions 1a, 1b and 1c on the Mono S column and their TPI activities are shown in Figure 4. Fractions 1a, 1b and 1c eluted at 0.139, 0.155 and 0.164 M NaCl, respectively, and all fractions possessed TPI activity as determined by the TNBS assay. However, in each case the TPI activity appeared to be distributed over a broad elution range and the activities in fraction 1a did not coincide with the absorbance at 280 nm. These results suggest the presence of at least two forms of the thiol protease inhibitors in extracts from dormant Artemia embryos.

Fractions 1a, 1b and 1c were then analyzed by SDS-urea gel electrophoresis and their molecular weights were determined. The results, illustrated in Figure 5, show that the TPI's in fractions 1a, 1b and 1c have molecular weights of 11.5, 12.2 and 12.6 kDa, respectively. Also, the presence of an additional small molecular weight protein in fraction 1c was observed.

Since the yields of TPI 1a, 1b and 1c from the Mono S column were not very large (see Table 2), subsequent analyses were performed on the total fraction 1.

Figure 4. Rechromatography of Mono S fractions 1a, 1b, and 1c on the FPLC column.

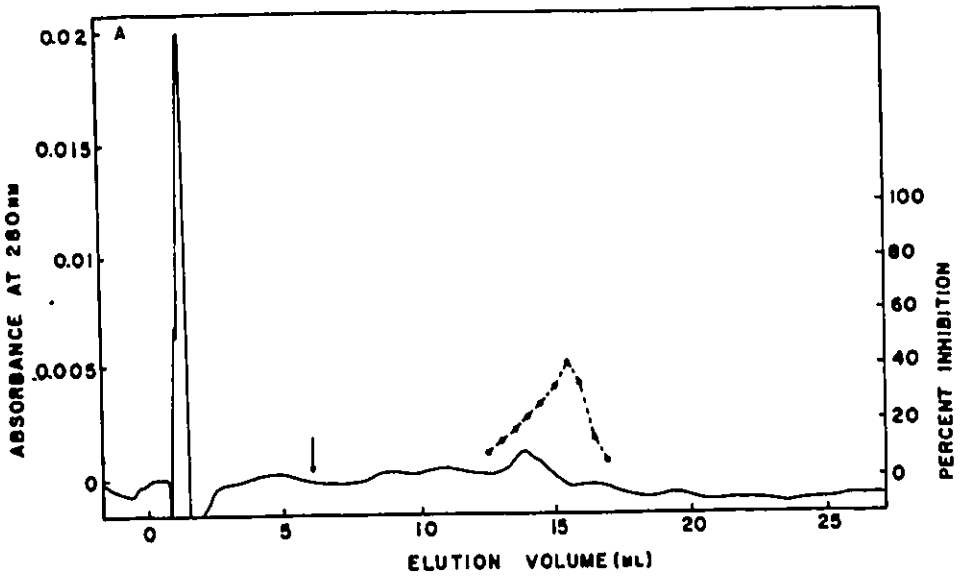
Panel A: Elution of fraction 1a from a Mono S column (20 µg was applied to the column).

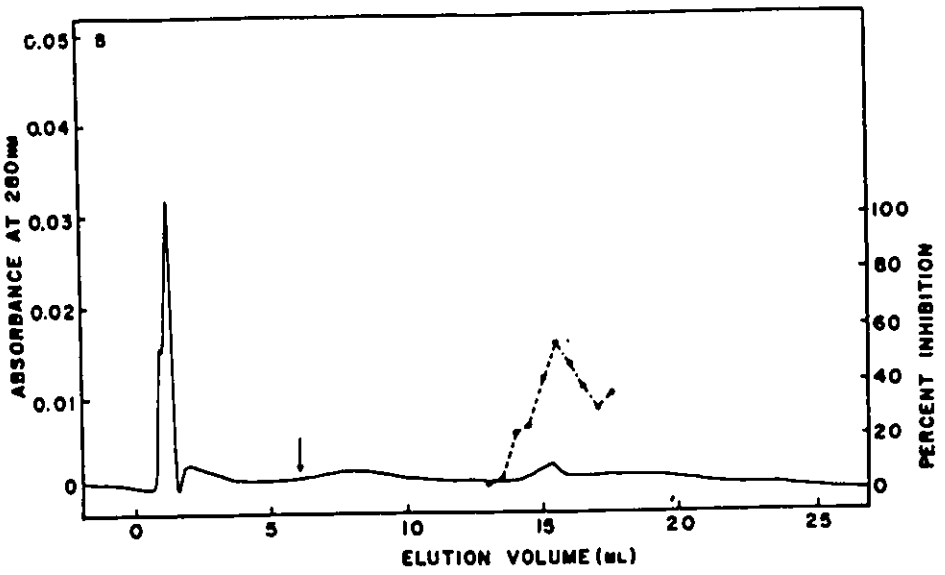
Panel B: Elution of fraction 1b from a Mono S column (26 µg was applied to the column).

Panel C. Elution of fraction 1c from a Mono S column (39 µg was applied to the column).

After a 6 ml wash in starting buffer (20mM sodium acetate, pH 5.0, containing 1 mM EDTA and 10% glycerol), the protein was eluted with a linear gradient (30 min.) of NaCl (to 0.3 M) in starting buffer at 1 ml/min. Protein was monitored at 280 nm and each fraction collected was assayed for TPI activity using the TNBS assay. The solid line represents the actual tracing from the recorder and the arrow indicates the beginning of the 30 minute gradient.

●--------●, inhibitor activity (as percent inhibition).


A
0.02
0.015
0.01
0.005
0
ABSORBANCE AT 280nm
100
80
60
40
20
0
PERCENT INHIBITION
0
5
10
15
20
25
ELUTION VOLUME (mL)


B
0.05
0.04
0.03
0.02
0.01
0
ABSORBANCE AT 280nm
100
80
60
40
20
0
PERCENT INHIBITION
0
5
10
15
20
25
ELUTION VOLUME (mL)

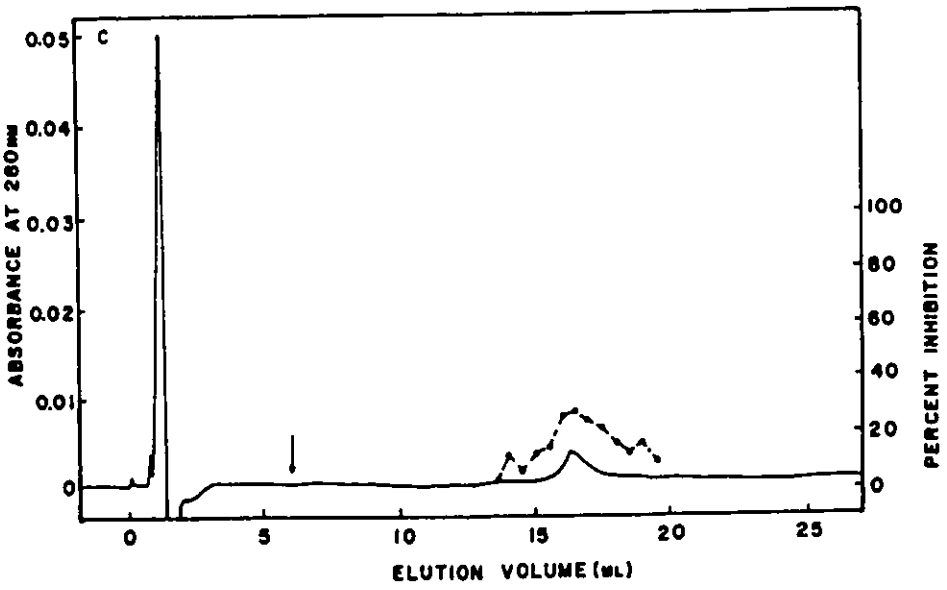

C
0.05
0.04
0.03
0.02
0.01
0
ABSORBANCE AT 280nm
100
80
60
40
20
0
PERCENT INHIBITION
0
5
10
15
20
25
ELUTION VOLUME (mL)

Figure 5. SDS-urea polyacrylamide gel electrophoresis of fractions 1a, 1b and 1c from the Mono S column.

Samples of each inhibitor preparation were electrophoresed on a 15% polyacrylamide gel containing 0.1M sodium phosphate, pH 7.2, 0.1% SDS and 6 M urea and stained with the silver protein stain.

Lanes 1 and 5 contain standard low molecular weight proteins: these are myoglobin backbone (17.0 kDa), fragments I and II combined (14.4 kDa), fragment I (8.1 kDa), fragment II (6.2 kDa), and fragment III (2.5 kDa). Lane 2 contains 0.64 µg of fraction 1a. Lane 3 contains 0.66 µg of fraction 1b and lane 4 contains 0.60 µg of fraction 1c.

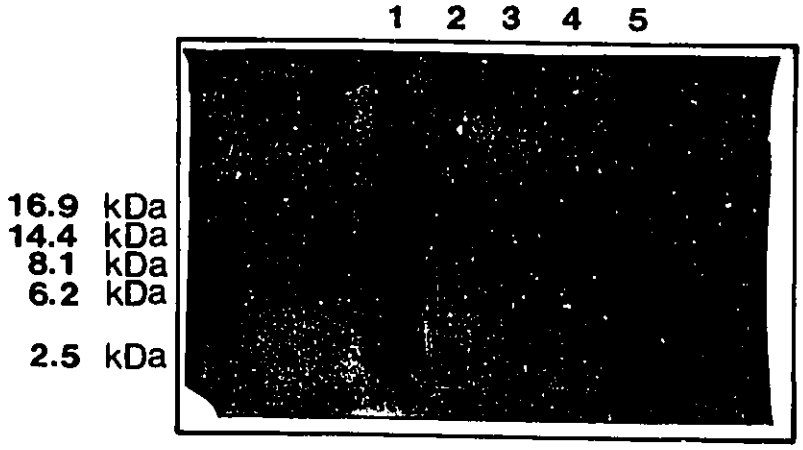

1 2 3 4 5
16.9 kDa
14.4 kDa
8.1 kDa
6.2 kDa
2.5 kDa

Table 2. Summary of Mono S purified thiol protease inhibitor fractions.[a]

Mono S inhibitor fraction	Protein (mg)	Specific inhibitor activity (mIU/mg)	Total inhibitor activity (mIU)	% of original activity
2nd Sephadex G-50	4.2	8,856	37,372	100
Inhibitor 1a	0.167	6,069	1,014	2.7
Inhibitor 1b	0.078	12,510	976	2.6
Inhibitor 1c	0.145	5,203	754	2.0
Ub-TPI's	0.279	5,079	1,417	3.8

(a) represents protein from 100 grams dry cysts as starting material.

The results of rechromatography of fraction 2 are shown in Figure 6. These results indicate the presence of one major protein peak possessing thiol protease inhibitor activity, and this protein eluted at 0.183 M NaCl. Amino acid sequencing of this protein following an additional purification step on a high performance liquid chromatography column (C-18) revealed that the protein is ubiquitin. The data and significance of this result will appear in the next section of the thesis.

The results obtained by cation exchange chromatography of the TPI preparation on the Mono S column showed poor yields and heterogeneity of potential inhibitors. Therefore, CM-cellulose chromatography of the Sephadex G-50 purified TPI preparation was attempted. The results of this experiment are shown in Figure 7. The column was equilibrated with 20 mM sodium acetate, pH 5.0, containing 10% glycerol, and the inhibitor preparation was equilibrated in the same buffer on a small Sephadex G-25 column. The sample was washed on the column with the above buffer and the proteins were eluted with 20 mM sodium acetate, pH 5, containing 10% glycerol and 50 mM KCl. After 150 ml had washed through the column, the protein remaining on the column was eluted with a linear gradient of 500 mM KCl.

The results in Figure 7 show that one major protein peak eluted with 50 mM KCl. Also apparent is the broad elution position of thiol protease inhibitor activity which

Figure 6. Rechromatography of Inhibitor fraction 2 on the Mono S column.

Fraction 2 from the first Mono S column (see Figure 3) was rechromatographed on a Mono S cation exchange column (0.5 x 5.0 cm). The solid line represents the actual recording from the monitor (A280). The broken line represents the thiol protease inhibitor activity as measured by the TNBS assay (●-------●). The arrow indicates the beginning of the 30 minute gradient.

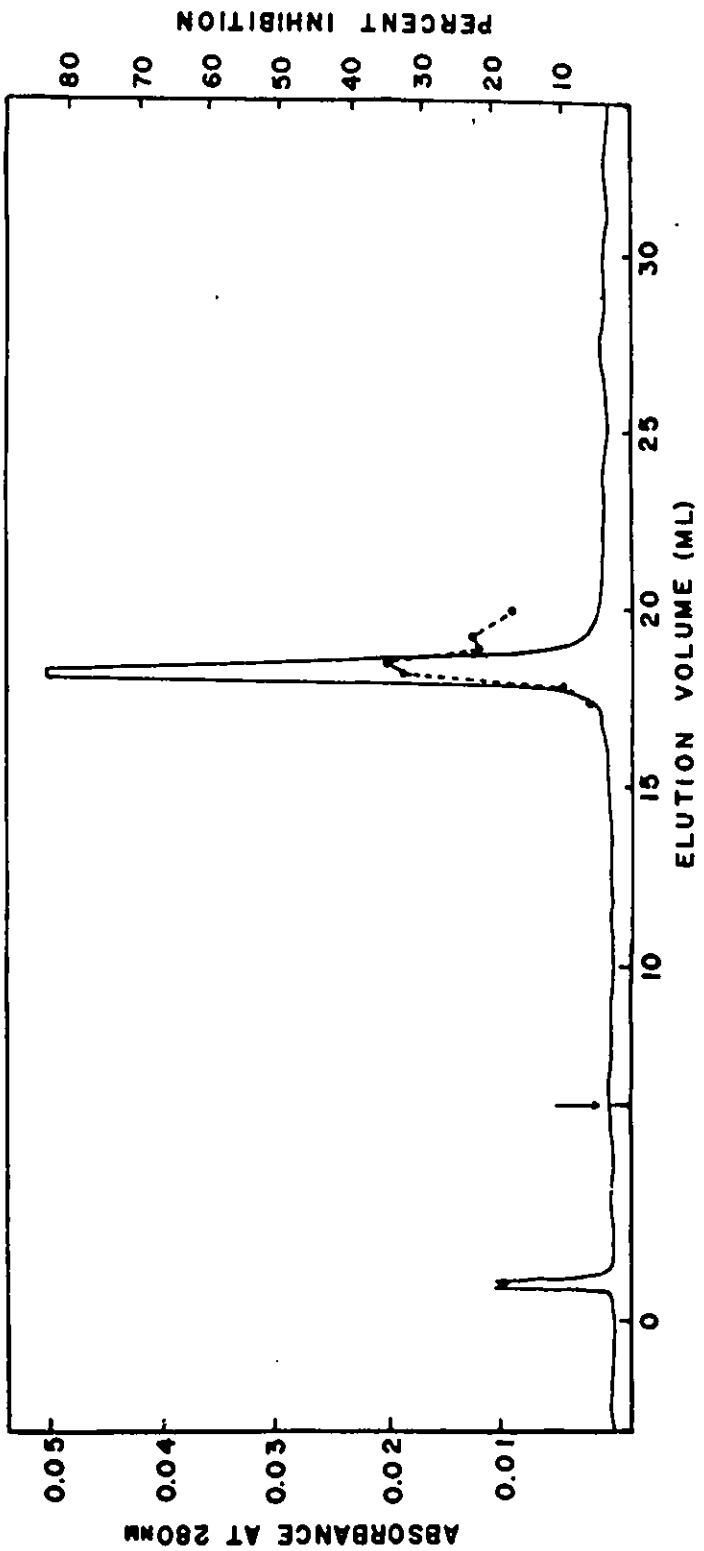
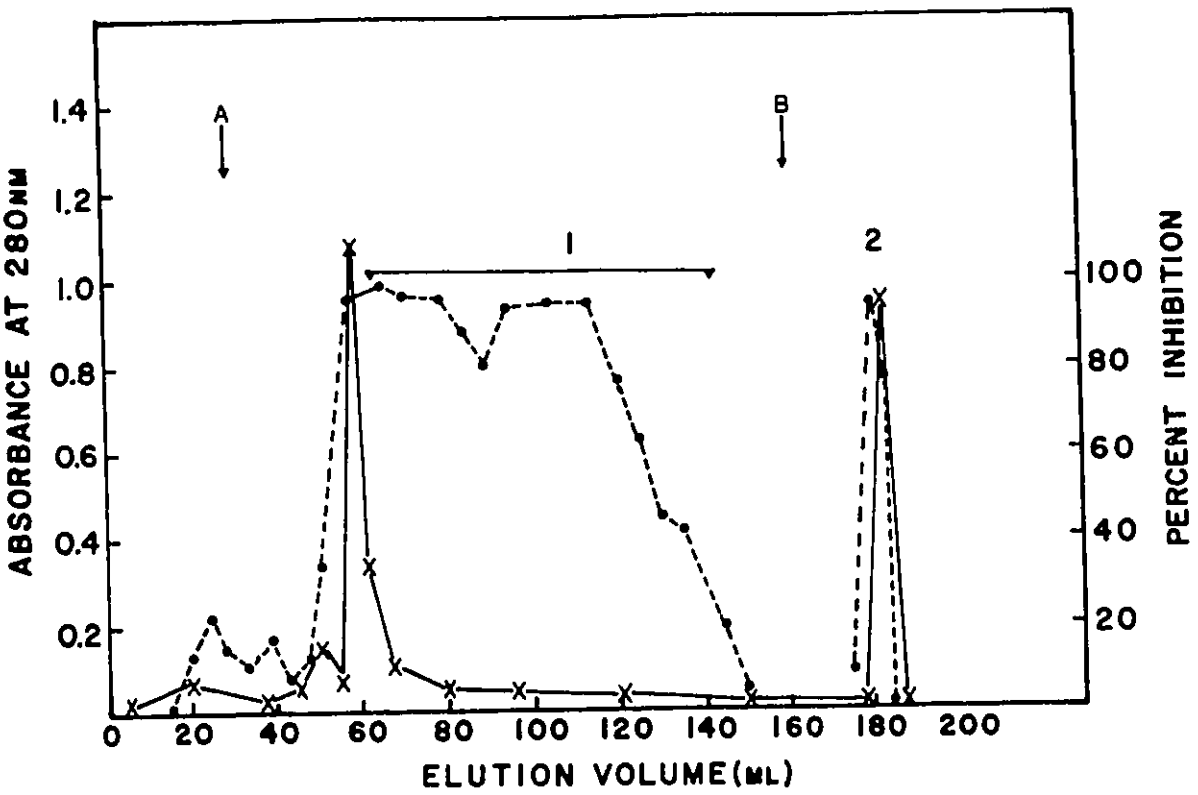

PERCENT INHIBITION
80
70
60
50
40
30
20
10
ELUTION VOLUME (ML)
0
10
15
20
25
30
ABSORBANCE AT 280nm
0.05
0.04
0.03
0.02
0.01

Figure 7. CM-cellulose chromatography of the thiol protease inhibitor preparation.

20.6 A_{280} units of a Sephadex G-50 purified thiol protease preparation were applied to a column of CM-cellulose (1 x 27 cm). Both the column and the TPI preparation were previously equilibrated with 20 mM sodium acetate, pH 5.0 containing 10% glycerol. The sample was washed on the column with the above buffer and the proteins were eluted with 20 mM sodium acetate, pH 5.0 containing 10% glycerol and 50 mM KCl (at A). After 150 ml had washed through the column, residual protein was eluted with a linear gradient of 500 mM KCl (at B).

●------●, inhibitor activity (as percent inhibition)

x------x, protein as measured by absorbance at 280 nm.


A
B
1
2
ABSORBANCE AT 280NM
PERCENT INHIBITION
ELUTION VOLUME(ML)
0
20
40
60
80
100
120
140
160
180
200
0.2
0.4
0.6
0.8
1.0
1.2
1.4
20
40
60
80
100

extends throughout several column fractions. The protease inhibitor was pooled as indicated and concentrated for further analyses (shown in the next section).

Also evident was a sharp protein peak which required higher salt concentrations to elute from the column. This protein was also found to possess a small amount of TPI activity. Therefore, it was also pooled and concentrated using Centricon-3 microconcentrators for further analyses.

C. High performance liquid chromatography of thiol protease inhibitor fractions from the Mono S column

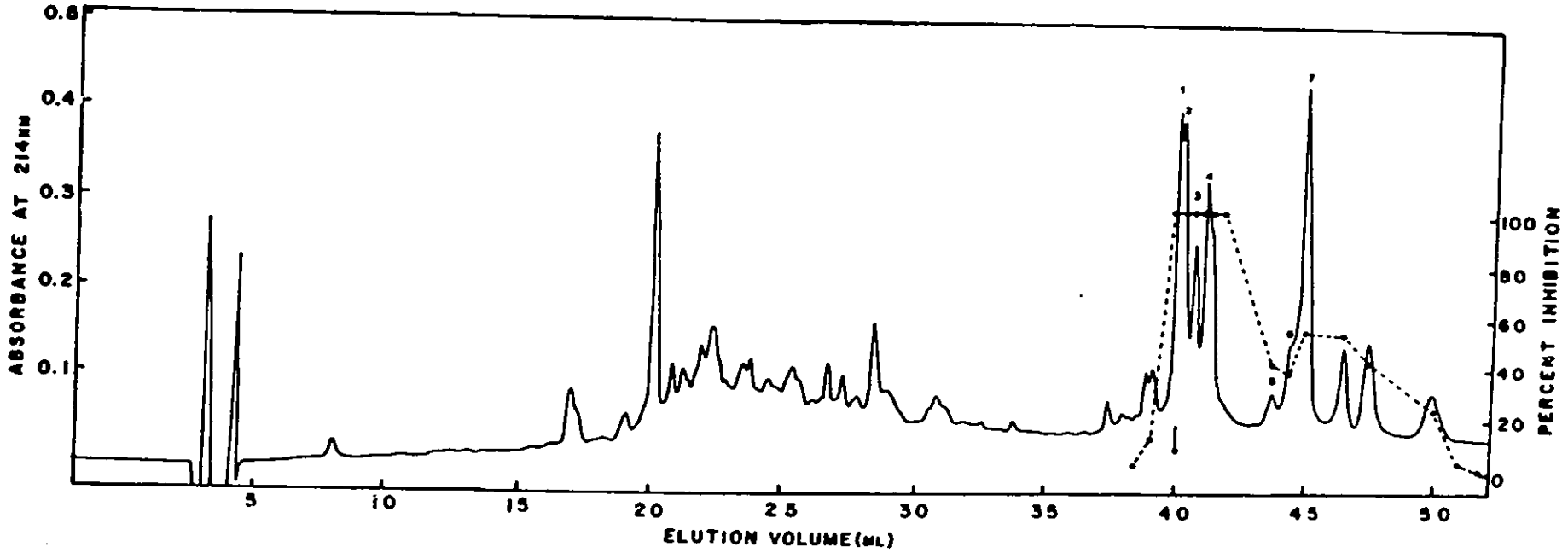
Fraction 1 from the CM-cellulose column (see Figure 7) was analyzed by high performance liquid chromatography on a C-18 reversed phase column, the results of which are illustrated in Figure 8. Each protein peak from the column was collected separately, lyophilized and subjected to a renaturation procedure (see Materials and Methods). Each renatured column fraction was assayed for thiol protease inhibitor activity using the TNBS assay. The results of these assays showed that at least four major UV-absorbing peaks possessed significant inhibitor activity. These were designated as inhibitor 1, 2, 3 and 4 in Figure 8. These results agreed with previous evidence for the presence of multiple protease inhibitors in dormant Artemia embryos. The HPLC results also suggest the presence of protease activity in the Sephadex G-50 fraction resulting in several peptide fractions becoming evident for the first time (vol. 15-30).

The next inhibitor fraction analyzed by HPLC was fraction 2 which eluted from the Mono S column at 0.183 M NaCl and showed considerable thiol protease inhibitor activity (see Figure 3). The results in Figure 9a show the presence of a large protein peak which eluted at 35% acetonitrile (at vol. 33.5 ml) followed by a cluster of three small peaks. Each peak was collected separately from the column, lyophilized and subjected to the renaturation procedure. After

Figure 8. High performance liquid chromatography of CM-cellulose purified protease inhibitors.

The TPI protein (68 µg) designated as fraction 1 in Figure 7 was applied to a C-18 reversed phase column (4.6 x 250 mm, Chemopack) previously equilibrated with 12% acetonitrile containing 0.1% TFA. The column was developed with a linear gradient (40 minutes) of acetonitrile to 36% acetonitrile containing 0.1% TFA (to the arrow) followed by isocratic elution with the 36% acetonitrile solution for an additional 12 minutes. The actual tracing from the recorder illustrates material that absorbs at 214 nm.

The TPI activity of this material is also shown as the broken line (●---------●). Potential thiol protease inhibitors are marked by numbers.


ABSORBANCE AT 214nm
0.6
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
45
50
ELUTION VOLUME(mL)
PERCENT INHIBITION
100
80
60
40
20
0

renaturation each fraction was assayed for thiol protease inhibitor activity and the results of this assay are shown (as bars) in Figure 9a. These results show that three minor protein peaks contain thiol protease inhibitor activity which were originally associated with the major protein peak. The major protein peak from the column, which also showed some inhibitor activity, was subjected to amino acid sequence analysis and found to be ubiquitin. The results of the amino acid sequence analysis are illustrated in Figure 10 and reveal 100% homology with human ubiquitin through the first 25 amino acids from the N-terminal end. Peaks 2 and 3 of the minor protein fraction were rechromatographed on the C-18 column (Figure 9b) and were found to elute at the same position as the ubiquitin-free thiol protease inhibitors (see Figure 8). The two major protein peaks possesed inhibitor activity. The first minor peak was not rechromatographed in this experiment because it was separable from the remaining minor peaks by collection.

The results from both FPCL and HPLC analyses suggest that dormant cysts of Artemia contain at least three thiol protease inhibitors, part of which are associated with ubiquitin. The significance of this asscciation in Artemia development will be discussed in a later section.

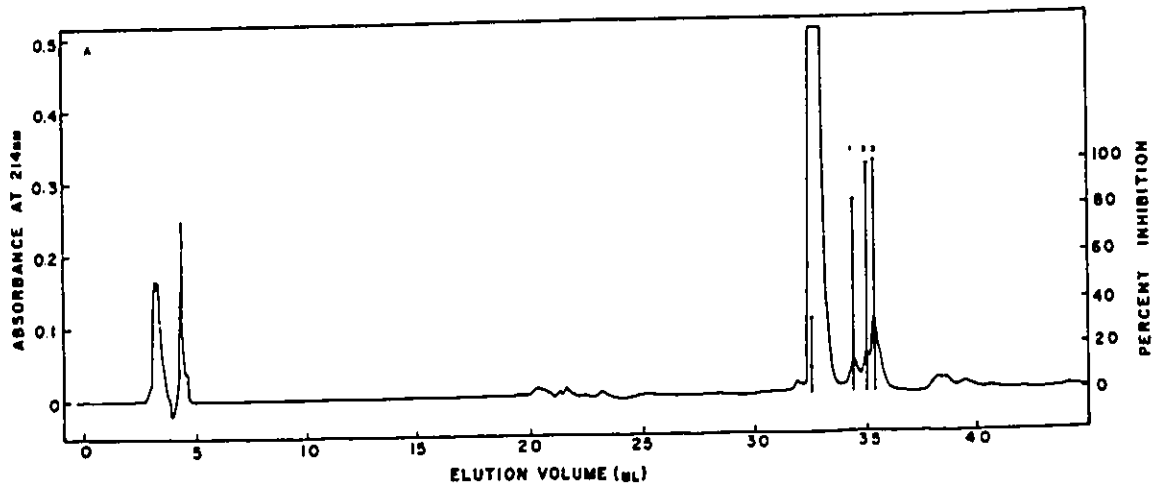
Figure 9. High performance liquid chromatography of protease inhibitors in Mono S column fraction 2.

Figure 9a. Mono S purified fraction 2 (41.7 µg) was applied to a C-18 reversed phase column (4.6 x 250 mm, Chemopack) which had previously been equilibrated with 12% acetonitrile containing 0.1% TFA. The column was developed with a linear gradient (40 minutes) of acetonitrile to 45% acetonitrile containing 0.1% TFA with a flow rate of 1 ml/min. The actual tracing from the recorder illustrates protein that absorbs at 214 nm.

Each peak was assayed for TPI activity and the results are shown as bars.

Figure 9b. Rechromatography of peak 2 and 3 inhibitors on an HPLC column. Combined peaks 2 and 3 from the HPLC column shown in Figure 9a were rechromatographed on the HPLC column using a shallow gradient. In this case the column was developed with a linear gradient (40 minutes) of acetonitrile to 36% acetonitrile containing 0.1% TFA.

Thiol protease inhibitor activity is illustrated as the broken line (●-------●).


A
ABSORBANCE AT 214nm
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
ELUTION VOLUME (mL)
100
80
60
40
20
0
PERCENT INHIBITION

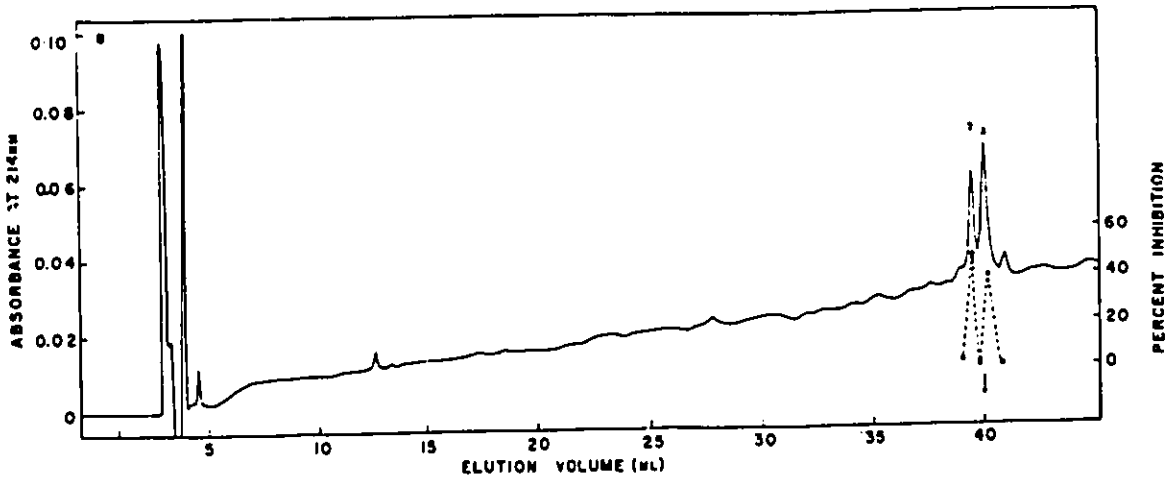

B
ABSORBANCE AT 214nm
0.10
0.08
0.06
0.04
0.02
0
5
10
15
20
25
30
35
40
ELUTION VOLUME (mL)
60
40
20
0
PERCENT INHIBITION

Figure 10. Amino acid sequence analysis of the major protein in Artemia Mono S fraction 2 compared to human ubiquitin.

	1				5				10				15				20				25
Artemia																					
Fraction 2:	M	Q	I	F	V	K	T	L	T	G	K	T	I	T	L	E	V	E	P	S	D	T	I	E	N
Ubiquitin:	M	Q	I	F	V	K	T	L	T	G	K	T	I	T	L	E	V	E	P	S	D	T	I	E	N

D. Further purification of the multiple thiol protease inhibitors designated as inhibitors 1-4 in Figure 8

The ubiquitin-free inhibitors designated as fractions 1, 2, 3 and 4 in Figure 8 were re-purified by HPLC rechromatography as illustrated in Figures 11 and 12. Figure 11 shows the rechromatography of inhibitors 1, 2, and 4 on a C-18 reversed phase column developed with a linear gradient (40 minutes) of acetonitrile to 36% containing 0.1% TFA. Each peak was collected, renatured and assayed for thiol protease inhibitor activity. The results of each assay are shown in Figure 11 as bar graphs. Each protein that was enriched by the process showed the most inhibitor activity in each case. The protein that was enriched in all four cases (inhibitors 1, 2, 3 and 4) was rechromatographed on the C-18 column. Figure 12 shows the results of the rechromatography of each of the inhibitors 1, 2, 3 and 4. Following this treatment, inhibitors 1, 2, 3 and 4 appeared to be relatively pure. Each was lyophilized, renatured and assayed for thiol protease inhibitor activity (shown as bars). As shown in Figure 12, only inhibitors 1 and 2 possessed significant thiol protease inhibitor activity. Inhibitors 3 and 4 may not have been fully renatured and for this reason were inactive.

Figure 11. Purification of the ubiquitin-free multiple thiol protease inhibitors by high performance liquid chromatography.

In all cases the C-18 reversed phase column was equilibrated with 12% acetonitrile containing 0.1% TFA. The column was developed with a linear gradient (40 minutes) of acetonitrile to 36% acetonitrile containing 0.1% TFA and then isocractically with the 36% acetonitrile solution beginning at the position of the arrow. In each panel the actual tracing from the recorder illustrates protein that absorbs at 214 nm and the TPI activity of each fraction is shown by a bar graph.

Panel A: Inhibitors 1 and 2 from Figure 8.
Panel B: Inhibitor 3 from Figure 8.
Panel C: Inhibitor 4 from Figure 8.

The large peaks eluting at the beginning of the gradient and at 17.5 ml were from materials in the renaturation buffer.

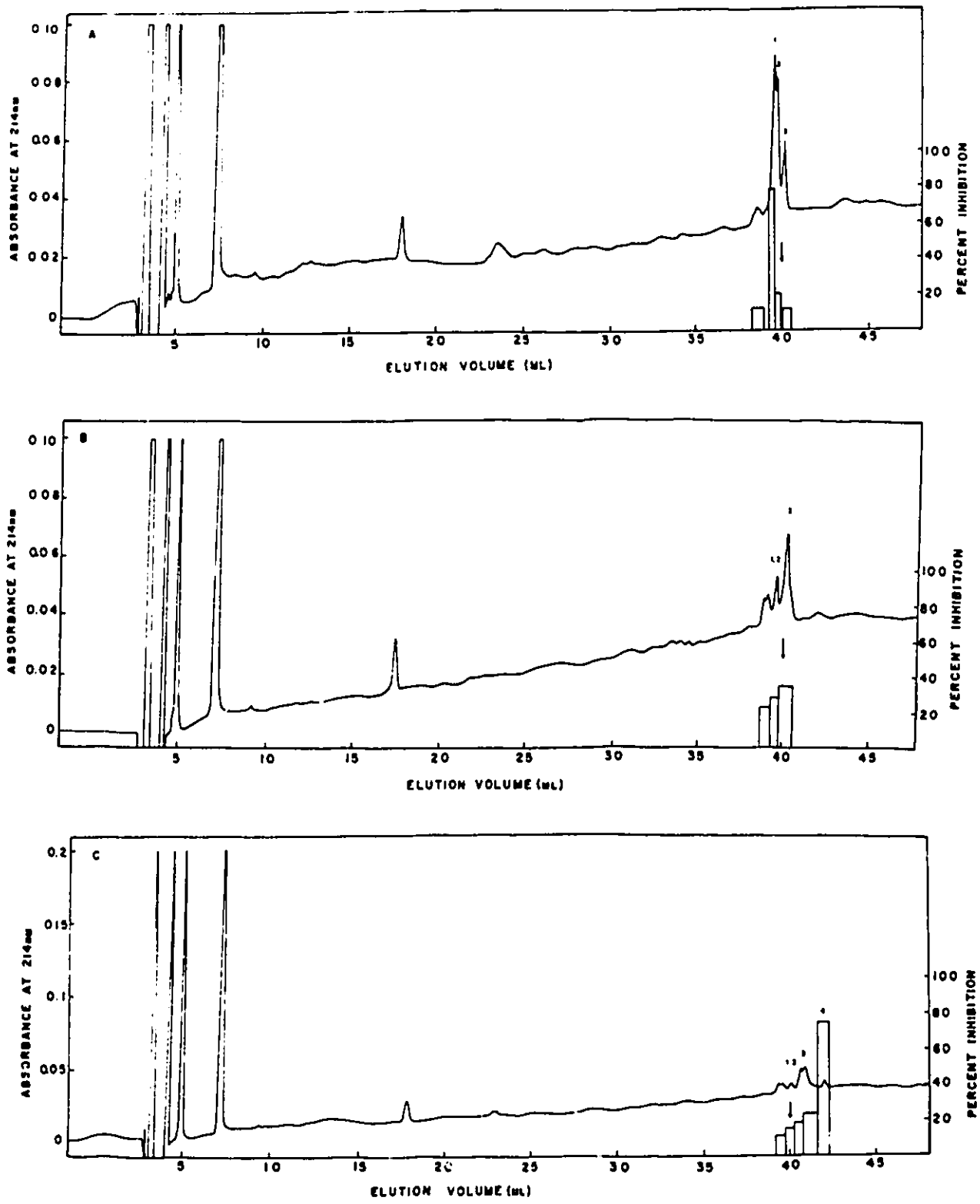
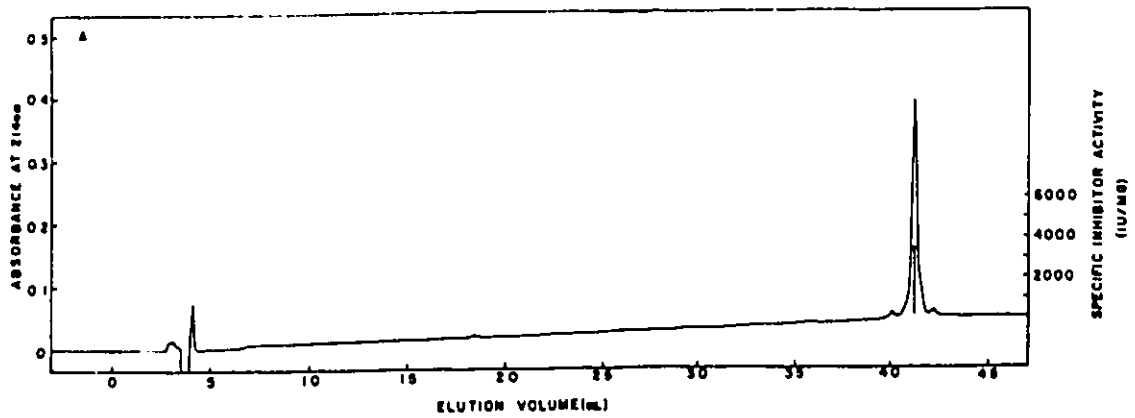
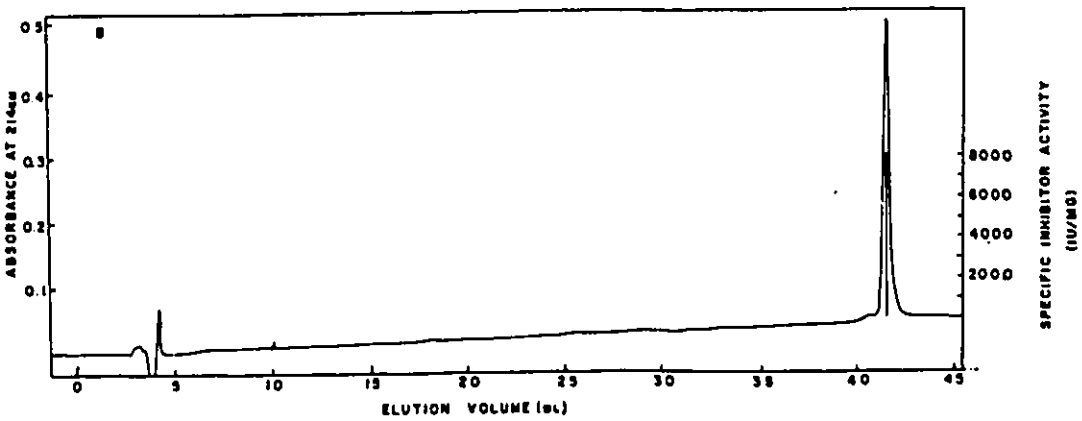

A
B
C
ABSORBANCE AT 214nm
PERCENT INHIBITION
ELUTION VOLUME (ML)
ELUTION VOLUME (mL)

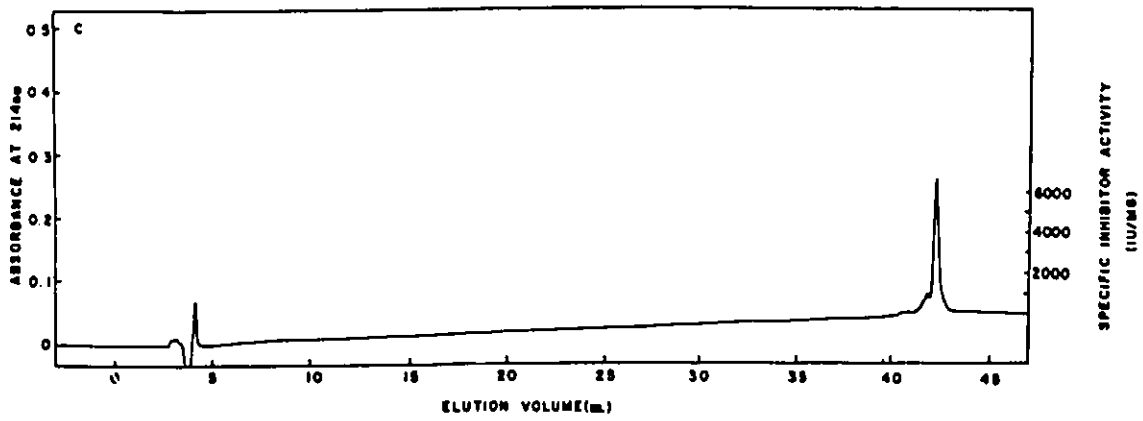
Figure 12. Rechromatography of ubiquitin-free thiol protease inhibitors by high performance liquid chromatography.

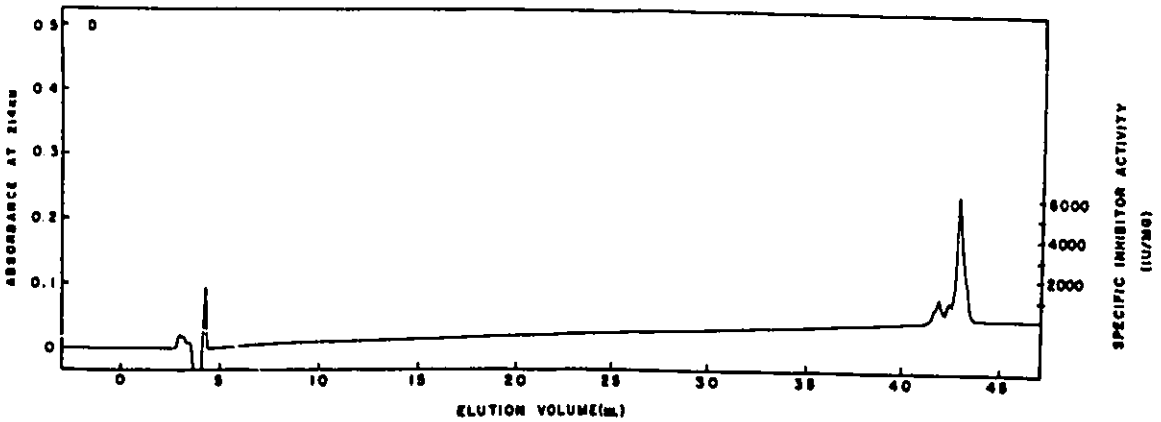
In all cases, the C-18 column was equilibrated with 12% acetonitrile containing 0.1% TFA. The column was developed with a linear gradient (40 minutes) of acetonitrile to 36% acetonitrile containing 0.1% TFA with a flow rate of 1 ml/min. After 40 minutes the protein were eluted isocratically with 36% acetonitrile and 0.1% TFA. In each panel the actual tracing from the recorder illustrates protein that absorbs at 214 nm. TPI activity is illustrated in Panel A and B as bars.

Panel A: Inhibitor 1 from Figure 11.
Panel B: Inhibitor 2 from Figure 11.
Panel C: Inhibitor 3 from Figure 11.
Panel D: Inhibitor 4 from Figure 11.


A
ABSORBANCE AT 214nm
SPECIFIC INHIBITOR ACTIVITY (IU/MG)
ELUTION VOLUME(mL)


B
ABSORBANCE AT 214nm
SPECIFIC INHIBITOR ACTIVITY (IU/MG)
ELUTION VOLUME (mL)


C
ABSORBANCE AT 214nm
SPECIFIC INHIBITOR ACTIVITY (IU/MG)
ELUTION VOLUME(mL)


D
ABSORBANCE AT 214nm
SPECIFIC INHIBITOR ACTIVITY (IU/MG)
ELUTION VOLUME(mL)

E. Sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis of purified thiol protease inhibitors

The migration properties of ubiquitin-associated thiol protease inhibitors on a polyacrylamide gel (15%) containing 0.1 M sodium phosphate, pH 7.2, 0.1% SDS and 6 M urea are shown in Figure 13 (panel A). The results show that ubiquitin has an average apparent molecular weight of 4.9 kDa and the associated inhibitors have an average apparent molecular weight of 12.3 kDa.

The ubiquitin-free thiol protease inhibitors purified by HPLC were analyzed similarly and the results show that inhibitors 1, 2, 3 and 4 were apparently pure (Figure 13, panel B). Based on migration distances of the inhibitors compared with standard protein markers (Figure 14), the apparent average molecular weights of inhibitors 1, 2, 3 and 4 were calculated to be 12.2, 12.2, 14.5 and 14.7 kDa, respectively.

Molecular weights were also determined by gel filtration on Sephadex G-50. A summary of the results is shown in Figure 15. These data show an average apparent molecular weight of 7.0 kDa for the thiol protease inhibitors. This was interesting, since ubiquitin itself has an apparent molecular weight of 5.5 kDa, while the inhibitors fall in the 12.2 kDa range.

These results show that at least a portion of the pool of Artemia embryo thiol protease inhibitor population is associated (non-covalently) with ubiquitin.

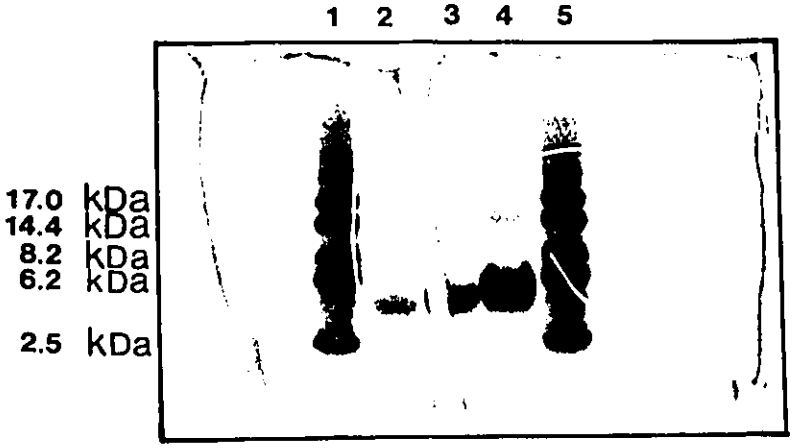
Figure 13. SDS-urea polyacrylamide gel electrophoresis of ubiquitin-associated and free TPI's.

Samples of each inhibitor preparation were electrophoresed on 15% polyacrylamide gels containing 0.1 M sodium phosphate, pH 7.2, 0.1% SDS and 6 M urea and stained with the silver protein stain.

Panel A: Ubiquitin-associated thiol protease inhibitors after Mono S chromatography (Figure 4). Lanes 1 and 5 contain the following standard low molecular weight proteins; myoglobin backbone (17.0 kDa), fragment I and II combined (14.4 kDa), fragment I (8.2 kDa), fragment II (6.2 kDa), and fragment III (2.5 kDa). Lanes 2 through 4 contain 0.215 µg, 0.644 µg, and 1.29 µg, respectively, of Mono S fraction 2 protein.

Panel B: Ubiquitin free thiol protease inhibitors after high performance liquid chromatography (Figure 12). Lanes 1 and 8 contain standard molecular weight proteins as described for Panel A above. Lanes 2 to 5 contain 1 ug each of inhibitors 1, 2, 3 and 4, respectively.

A


1 2 3 4 5
17.0 kDa
14.4 kDa
8.2 kDa
6.2 kDa
2.5 kDa

B

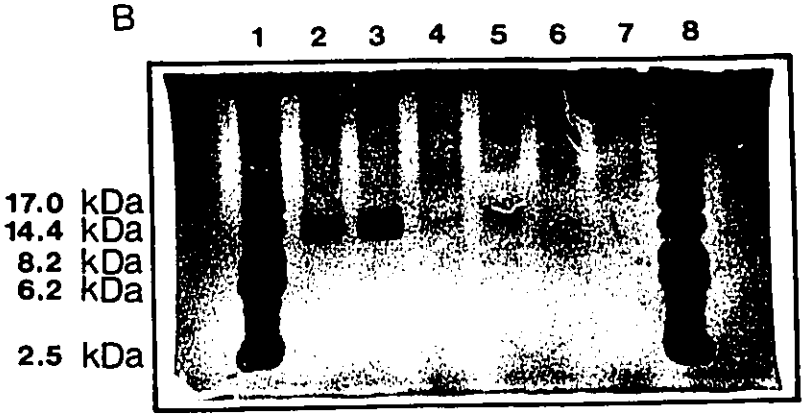
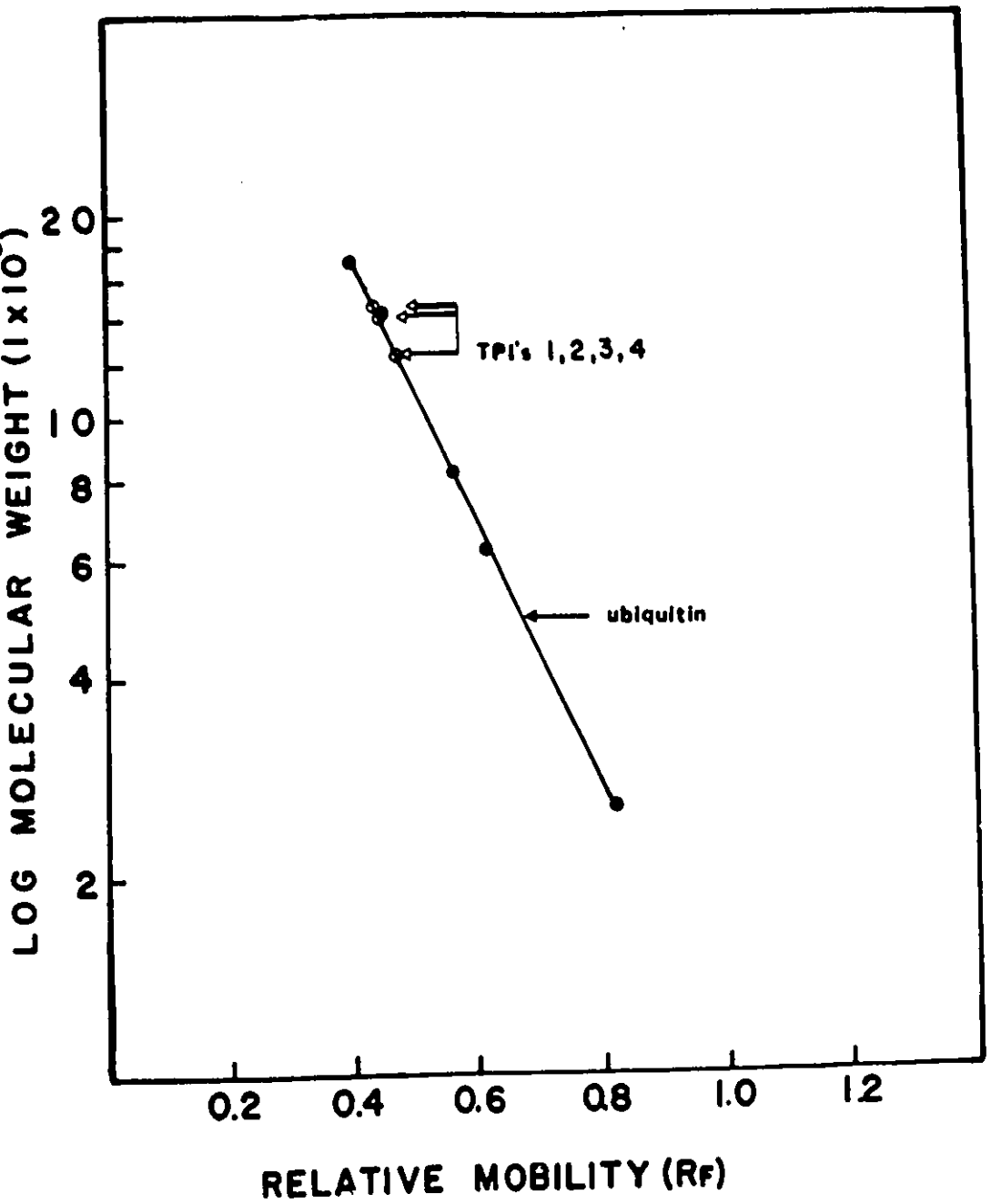

1 2 3 4 5 6 7 8
17.0 kDa
14.4 kDa
8.2 kDa
6.2 kDa
2.5 kDa

Figure 14. Molecular weight determination of ubiquitin-associated and free TPI's by SDS-urea polyacrylamide gel electrophoresis. Standard protein markers used were all fragments of myoglobin: myoglobin backbone (17.0 kDa), fragments I and II combined (14.4 kDa), fragment I (8.2 kDa), fragment II (6.2 kDa) and fragment III (2.5 kDa). The average electrophoretic position of each inhibitor on the gel compared to the standard proteins is indicated by the arrow.


LOG MOLECULAR WEIGHT (1x10^3)
20
10
8
6
4
2
TPI's 1,2,3,4
ubiquitin
0.2
0.4
0.6
0.8
1.0
1.2
RELATIVE MOBILITY (Rf)

F. Kinetic analysis of the ubiquitin-associated thiol protease inhibitors

The mechanism of inhibition of the Artemia thiol protease by the ubiquitin-associated thiol protease inhibitors was determined. The reaction mixtures containing varying amounts of inhibitor and substrate (protamine sulfate) with a constant amount of purified Artemia thiol protease (0.6 µg) in a buffer containing 0.1 M sodium acetate, pH 5.0, 10% glycerol, 1 mM EDTA and 1 mM DTT.

The results of the kinetic studies are illustrated in Figure 16 and show that the ubiquitin-associated inhibitors are uncompetitive inhibitors. The K_m for the control reaction was calculated to be 9.88×10^{-5} M using an average mass of 7.5 kDa for the substrate protamine sulfate. The K_i for the ubiquitin-associated TPI's was calculated to be 9.1×10^{-9} M using an average molecular weight of 12.3 kDa for the inhibitors. The kinetics of inhibition of the purified free TPI's were not done in these experiments because of insufficient material and the fact that data on the K_i of the combined free inhibitors was obtained previously (Warner, 1989).

Figure 15. Molecular weight determination of TPI preparations by gel filtration on Sephadex G-50.

A Sephadex G-50 column (superfine, 0.75 x 42 cm) was equilibrated with 15 mM potassium phosphate, 25 mM potassium chloride and 10% glycerol. 4.4 mg of a Sephadex G-50 purified TPI preparation was applied to the column and the elution volume of the TPI activity determined compared to the elution volume of the following molecular weight marker proteins: soybean trypsin inhibitor (20.1 kDa), RNase (13.7 kDa), and aprotinin (6.2 kDa). The K_{av} of the TPI preparation was calculated and compared to the K_{av} of the standard proteins. The arrow indicates the K_{av} of the TPI preparation giving an average molecular weight of 7 kDa. The void volume of the column was determined using dextran blue.

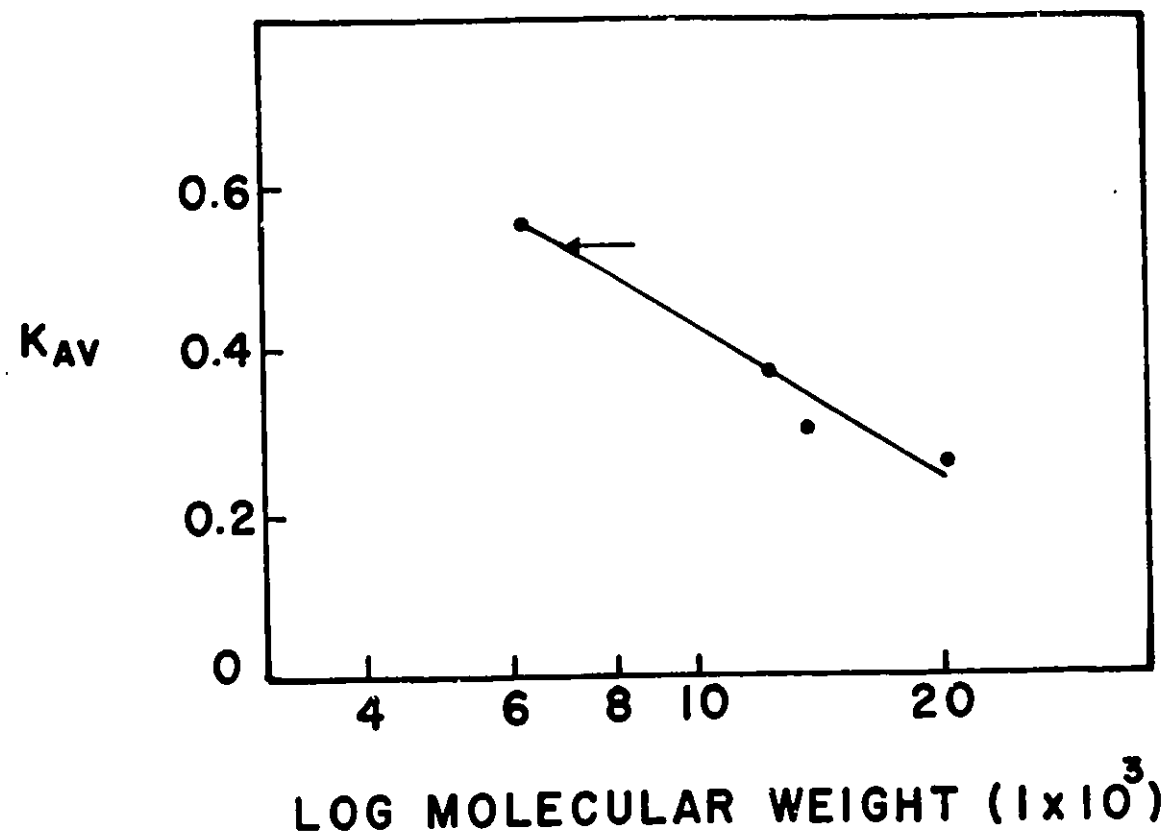

K_{AV}
0.6
0.4
0.2
0
4
6
8
10
20
LOG MOLECULAR WEIGHT (1×10^3)

Figure 16. Kinetic analysis of ubiquitin-associated thiol protease inhibitors.

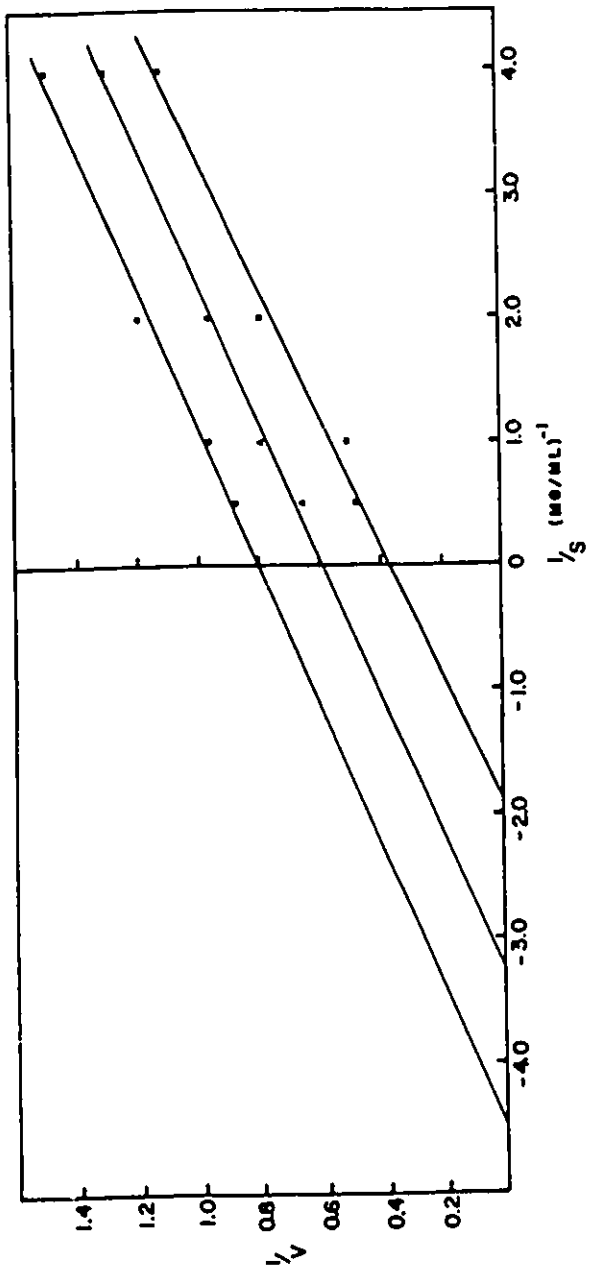
Each reaction vessel contained 0.6 ug of purified Artemia thiol protease, protamine sulfate as substrate and

either buffer (■------■),

0.6 µg TPI preparation (▲------▲)

or 1.0 µg TPI preparation (●------●)

in a standard reaction vessel at pH 5.0. The thiol protease inhibitor preparation was from the Mono S cation exchange column (fraction 2, Figure 5). The temperature of the reaction was 30°C. The equation for all three slopes was generated by regression analysis of all data points in each set. An average mass of 7.5 kDa was used as the molecular weight for protamine sulfate to calculate the kinetic constants.


1/V
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-4.0
-3.0
-2.0
-1.0
0
1.0
2.0
3.0
4.0
1/S (MG/ML)⁻¹

IV. DISCUSSION

There is now a considerable body of evidence that regulation of intracellular protease activity by endogenous protease inhibitors is essential for maintaining cell viability and preventing inappropriate proteolysis. This evidence has been gathered from studies at the cellular, tissue and whole animal levels. Examples of the importance of proteolytic regulation include: the degradation of the protein B-amyloid by proteases into potential Alzheimer's causing agents (Kang et al., 1987 and Tanzi et al., 1987), the regulation of muscle degrading proteases in muscular dystrophy (Gopalan and Warner, 1987), the regulation of proteases involved in metastasis (Bond and Butler, 1987) and the suppression of the transformation of cells by ras oncogenes (Frech et al., 1990). These examples give merit to the critical importance of proteolytic regulation in biological systems.

Furthermore, proteases have been implicated in the regulation of developmental events such as the activation of inactive messenger ribonucleoproteins (mRNP's) (Spirin, 1966), the activation of metabolically repressed ribosomes (Metafora, 1971) and yolk utilization (Warner and Shridhar, 1985; Perona and Vallejo, 1985).

In dormant Artemia embryos, thiol protease activity is regulated by at least two types of low molecular weight thiol protease inhibitors (TPI's) present in the cytosol, one which is dialyzable and the other is non-dialyzable (Na-

gainis and Warner, 1979; Warner and Shridhar, 1985). During the first 6 to 9 hours after resumption of development after dormancy, the activity of the non-dialyzable TPI's increases by approximately 60% and then declines to undetectable levels over the remainder of the developmental process (Warner, 1987). Moreover, as the TPI activity decreases, there is an apparent increase in thiol protease activity. This observation suggests that the thiol protease inhibitor gene(s) may be developmentally regulated in Artemia by stage-specific transcriptional or post-transcriptional control mechanisms.

For this reason, the thiol protease inhibitors were extracted from dormant Artemia embryos, purified and analyzed as discussed in this section of the thesis.

Fast protein liquid chromatography of Sephadex G-50 purified TPI preparations from dormant Artemia embryos resolved approximately six fractions containing TPI activity. This finding suggested the presence of a complex mixture of (multiple forms) thiol protease inhibitors. Due to this complexity, fractions containing the most activity were selected for further analyses. These fractions included fraction 1 and 2 from the Mono S column (see Figure 3). Fraction 1 consisted of approximately three protein peaks eluting at 0.139, 0.155 and 0.164 M NaCl on the Mono S column while fraction 2 consisted of an abundant protein peak eluting at 0.183 M NaCl.

SDS-urea polyacrylamide gel electrophoresis (PAGE) was performed to better resolve the low molecular weight proteins. The three peaks in fraction 1 were analyzed separately by this technique and found to have molecular weights of approximately 11.5, 12.2 and 12.6 kDa respectively (see Figure 5). Since the final yield of each peak was low (see Table 2), the three peaks were pooled and subsequently analyzed together. These results suggested the presence of at least three distinct forms of the thiol protease inhibitor extracted from dormant Artemia cysts. Each form was slightly different in ionic charge at pH 5.0. One experiment on isoelectric focusing was performed on these inhibitors separately and they all had pI's between 5.0 and 5.4 (data not shown). These pI values are in accordance with those found previously for the same inhibitors (Warner, 1987).

The Artemia TPI's from fraction 1 resemble human cystatins in size as their molecular weights are in the range of 11.0 to 13.5 kDa and they exist in multiple forms (Barrett, 1985). Isoelectric points for non-human cystatins are usually located in the range of pH 5-7, which is consistant with the pI's for the Artemia TPI's. Turk et al. (1985) succeeded in isolating chicken cystatin and showed that this inhibitor exists in several isoelectric forms of which the two major forms (A and B) have pI's of 5.6 and 6.5. Form B contained 116 amino acid residues and was found to represent the elongated form of A by 8 amino acids at the N-terminus.

These researchers suggested that form A may be the result of proteolytic cleavage of form B during the purification procedure. Other researchers have documented the presence of seven isoforms of a low molecular weight cysteine protease inhibitor from human amniotic fluid (Rohrlich et al., 1985). Some researchers attribute the presence of multiple isoforms of an inhibitor to artifacts created by heat treatment used in the purification procedure (Kominami et al., 1982). However this treatment was not used in the purification of the TPI's from dormant Artemia embryos. In addition, other researchers have suggested that degradation of part of the amino-terminal end of the inhibitor protein (by limited proteolysis), produces forms lacking one or more amino acid residues, as the reason for multiple inhibitor forms.

Previous studies on partially purified TPI preparations from dormant Artemia embryos (Warner, 1989; Warner, 1987), revealed that the TPI's exhibited non-competitive inhibition, with a K_i value of 1.7×10^{-11}M (Warner, 1989). These partially purified preparations were mainly free of ubiquitin-associated TPI's. Results presented in this thesis show that the K_i for ubiquitin-associated TPI preparations is 9.1×10^{-9} M and that inhibition was uncompetitive (see Figure 16). Most low molecular weight thiol protease inhibitors exhibit either competitive or non-competitive inhibition and have K_i values in the range of 5×10^{-12} M to 1.2×10^{-10} M (Kominami et al., 1982; Barrett, 1985; and

Waxman, 1978). Kinetic studies were not performed on the purified TPI's free of ubiquitin in this thesis due to low inhibitor yields.

Studies on partially purified Artemia TPI preparations (Warner, 1989) also revealed that the inhibitors are less stable to pH and heat than most mammalian intracellular thiol protease inhibitors. The Artemia inhibitors were found to be stable between pH 6.0 and 9.5 (at 40°C) and unstable below pH 5.0. In the results presented in this thesis, TPI preparations were found to lose activity at pH 5.0 and since cation exchange chromatography was performed at this pH, protective measures were taken to raise the pH during the procedure.

Fraction 2 from the Mono S column eluted at 0.183 M NaCl following the elution of the first three TPI's. This fraction also possessed significant TPI activity and was present in large quantities (approximately 27% of the total protein eluting from the column) relative to those in fraction 1. Rechromatography of this major protein peak on the Mono S column showed the presence of only one major peak eluting at 0.183 M NaCl and possessing TPI activity. However, when this peak was analyzed by HPLC, one major and three minor protein peaks appeared. At this point in my experiments, the renaturation procedure had not yet been utilized and it was assumed that the major peak was correlated with the TPI activity. Therefore, this major protein peak was subjected to cyanogen bromide fragmentation and

amino acid sequencing. The sequencing results revealed that the protein was identical to human ubiquitin (see Figure 10) suggesting that the three minor protein peaks were the potential thiol protease inhibitors. Samples of bovine RBC and baker's yeast ubiquitin were analyzed and found to be devoid of inhibitor activity confirming the fact that ubiquitin is not a thiol protease inhibitor.

When the ubiquitin peak from the Mono S column was analyzed by SDS-urea PAGE, two protein bands appeared upon silver staining. One band had an average molecular weight of 4.9 kDa and was identified as ubiquitin. The other protein band possessed an average molecular weight of 12.3 kDa, which was the same average molecular weight as the three TPI peaks in fraction 1. This finding suggested the presence of ubiquitin-associated TPI's. Molecular weight estimates by gel filtration show that ubiquitin has a molecular weight of 8.5 kDa (Schlesinger et al., 1975) and on highly cross-linked gels a molecular weight of 5.5 kDa (Ciechanover et al., 1978). The position in which the thiol protease inhibitor activity eluted from gel filtration columns was very broad. This observation suggested the presence of multiple forms of the TPI's.

Rechromatography of the TPI fraction on Sephadex G-50 resulted in a slight separation of two fractions (see Figure 1). The average molecular weight of these fractions was determined to be 7.0 kDa. This result was perplexing because ubiquitin itself is known to have an average molecular

weight of 8.0 kDa daltons by gel filtration (Rechsteiner, 1988). The purified TPI's have an average molecular weight of 12.3 kDa and ubiquitin from Artemia embryos was found to have a molecular weight of 4.9 kDa by SDS-urea PAGE. I believe that the average molecular weight of the TPI's of 7.0 kDa indicates anomolous behavior of these proteins as observed by other researchers over the years.

The presence of ubiquitin-associated and free TPI's in extracts from dormant Artemia embryos was further investigated by high performance liquid chromatography. HPLC analysis revealed that the TPI's from fraction 1 eluted in the same position as the ubiquitin-associated TPI's from fraction 2 (see Figure 9a and 9b).

Later studies included the use of a renaturation procedure after HPLC analysis of TPI preparations. This procedure involved denaturation of protein fractions in 6M guanidine-HCl and subsequent renaturation in 20 mM Tris-HCl, pH 7.9 containing 0.2M EDTA, 10mM B-mercaptoethanol, 20% glycerol and 0.1M KCl. This procedure proved extremely useful in the identification of thiol protease inhibitor fractions from the reversed phase (C-18) HPLC column. By this method, at least four major TPI's were identified from ubiquitin-free preparations (see Figure 8). However, when each TPI was purified to apparent homogeneity by HPLC, complete renaturation of all fractions did not occur which makes exact identification difficult.

The renaturation procedure also proved that the three ubiquitin-associated minor protein peaks were TPI's. These TPI's eluted in the same position on the reversed phase column as the ubiquitin-free TPI's. Therefore, the "free" and "bound" inhibitors were identified as one and the same inhibitors. Therefore, cation exchange chromatography separates free and ubiquitin-associated low molecular weight TPI's from preparations of dormant Artemia embryos.

Ubiquitin is a 76 residue protein which is present in eukaryotes free or covalently joined by its carboxyl-terminal glycine residue to various proteins (Chau et al., 1989). This small abundant protein plays a central role in intracellular proteolysis and this function is extremely important due to the extreme conservation of its sequence.

One function of ubiquitin is to mark proteins destined for degradation by covalent conjugation to a targeted proteolytic substrate (see Introduction). It has also been suggested that ubiquitin reversibly joins to an acceptor protein thereby modulating protein function without destabilizing the acceptor protein (Chau et al., 1989). This hypothesis accounts for the existence of metabolically stable ubiquitin-protein conjugates which may be the case with the TPI's in dormant Artemia embryos.

Covalent conjugation of ubiquitin to protein is by an isopeptide bond between the carboxyl-terminal glycine residue of ubiquitin and the epsilon-amino group of a lysine residue in an acceptor protein (Chau et al., 1989). Chau et

al. (1989) showed that all ubiquitin moieties in a targeted short-lived modified substrate occur as a multi-ubiquitinated chain where the carboxyl-terminal glycine^{76} of one ubiquitin is joined to lysine^{48} of an adjacent ubiquitin. Their results suggested that the multi-ubiquitinated chain in a targeted protein is essential for protein degradation. Mono-ubiquitination of a protein is not enough for its degradation. They hypothesize that the multi-ubiquitinated chain provides binding sites for the ubiquitin-dependent protease which degrades a ubiquitinated proteolytic substrate. This may be the reason why mono-ubiquitinated intracellular proteins are metabolically stable.

Researchers have documented the existence of ubiquitin conjugates in the cell nucleus (Busch and Goldknopf, 1981) and on the cell surface (Siegelman et al., 1986 and Yarden et al., 1986) both of which are sites where proteolytic function for ubiquitin is unlikely. Furthermore, it has been found that DNA-associated histones H2A and H2B are not destabilized by ubiquitination *in vivo* (Finely and Varshavsky, 1985). Therefore, conjugation of ubiquitin to proteins such as the *Artemia* low molecular weight TPI's may serve a regulatory role. These conjugates could undergo a non-degradative disassembly allowing reversibility to the regulatory modification by ubiquitin.

In *Artemia* the TPI activity increases during the first six to nine hours of development after dormancy and then declines rapidly over the next 24-27 hours (Warner, 1987).

As well, ubiquitin mRNA reaches a maximum five hours after resumption of development and then rapidly declines (Sastre et al., 1989). Whether this coincidence is significant in the reversible regulation of the Artemia low molecular weight TPI's by ubiquitin remains to be determined. However, in such an hypothesis, it is possible that ubiquitin regulates the low molecular weight TPI activity during dormancy by conformational modification.

Perhaps the association of the TPI's with ubiquitin signifies one stage in their degradative pathway. To determine whether this is true, the nature of the amino-terminal residue of each TPI must be determined as the N-end rule dictates.

SDS-urea polyacrylamide gel electrophoresis of Mono S column fractions (data not shown) revealed the presence of a 27 kDa protein only in certain fractions (results not shown). The fractions were obtained and separated as follows. One Sephadex G-50 step was performed and the resultant active fractions were divided into region A (TPI fractions eluting first) and region B (TPI fractions eluting second containing less protein). This process was repeated three times for each region. Then, regions A and B were separately analyzed by cation exchange chromatography, SDS-urea PAGE and HPLC.

By cation exchange chromatography, region A was found to be mainly composed of fractions 3 and 4, while fraction 2 (containing ubiquitin) was minimal (see Figure 3). SDS-urea

PAGE of the total protein in region A showed the presence of a dominant protein with a molecular weight of 27 kDa with minimal amounts of ubiquitin and the TPI fractions. SDS-urea PAGE of Mono S purified fractions revealed that fraction 2 contained ubiquitin as the dominant protein and minimal amounts of the TPI's. Fraction 3 was similar in composition to fraction 2. Fraction 4 contained the 27 kDa protein as the dominant component. Fraction 2 possessed TPI activity whereas fractions 3 and 4 possessed less activity in Mono S column fractions.

From these results one may hypothesize that the 27 kDa protein may be a multi-ubiquitin-TPI complex. This complex may be partially dissociated by cation exchange chromatography at pH 5.0, resulting in mono-ubiquitins and free TPI's observed in fraction 2 and releasing TPI activity.

SDS-urea PAGE of the total Mono S region B revealed ubiquitin as the dominant protein, a significant amount of the TPI fraction, and a smaller amount of the 27 kDa protein. Fraction 1 contained a significant amount of the TPI fraction and a minor amount of ubiquitin. Fraction 2 contained mainly ubiquitin and the TPI fraction. Fraction 3 contained minor amounts of ubiquitin, significant amounts of TPI and the 27 kDa protein.

HPLC analysis of each of the above fractions confirmed the observations by SDS-urea PAGE. The TPI proteins eluted from 39% to 41% acetonitrile in each active fraction, which was in accordance with previously determined elution positions (see Figure 9).

In one experiment, fraction 2 (ubiquitin) from the Mono S column was rechromatographed on the same column. Fraction 2 was the dominant protein peak as expected, however, fraction 4 reappeared and both fractions contained TPI activity. As discussed above, fraction 4 contains the 27 kDa protein. This may signify the formation of a multi-ubiquitin-TPI complex with a weight of 27 kDa, composed of the TPI fraction (12.3 kDa) and approximately three ubiquitin monomers (14.8 kDa). This complex would possess an overall mass of 27.1 kDa.

These results present a case for multi-ubiquitin-TPI formation in dormant Artemia embryos and explains the occurrence of dynamic results. This is, however, circumstantial evidence which requires further experimentation.

Ubiquitin-dependent proteolysis is a complex process involving multiple proteases and ATP. In order for this process to be occurring in Sephadex G-50 extracts from dormant Artemia embryos, all the necessary machinery must be present. The enzymes involved in ubiquitin-dependent protein degradation have the following molecular weights; E1

(200 kDa), E2 (25 kDa), and E3 (250 kDa). Therefore, it seems unlikely that these processes are occurring in Sephadex G-50 TPI preparations.

Another explanation for the presence of multiple TPI fractions is by the action of proteolytic activity. CM-cellulose chromatography was performed on Sephadex G-50 purified TPI fractions as an alternative to the Mono S column. One fraction containing TPI activity was then analyzed by HPLC as was shown in Figure 8. HPLC resulted in the separation of at least four ubiquitin-free TPI's. The presence of smaller molecular weight fragments was observed, eluting from 17 ml to 30 ml on the reversed phase column which did not contain TPI activity. These fragments may have been the result of proteolytic degradation of the TPI's. This would also explain the relatively low yields of TPI's obtained during purification of the inhibitors from extracts of dormant Artemia embryos (see Table 2 and 3). Fraction 2 from the CM-cellulose column (see Figure 7) which eluted at 500 mM KCl and possessed TPI activity was also analyzed by HPLC (data not shown). The results showed the presence of multiple fractions active in TPI activity throughout the elution volume but most proteins were very hydrophobic and tightly bound to the column.

These results indicate that Sephadex G-50 TPI preparations from dormant Artemia embryos contain multiple TPI's or TPI fragments which are active and which bind to a CM-cellulose column at pH 5.0. Some of these proteins require

high salt concentrations to elute while others require low salt concentrations to elute. Either there is an overabundance of low molecular weight thiol protease inhibitors in dormant embryos or proteolytic fragmentation is occurring to generate multiple forms.

The results raise some important questions regarding the state of thiol proteases and thiol protease inhibitors in dormant Artemia embryos. It has been found that in crude homogenates of dormant embryos and early gastrulae of Artemia, a thiol protease is responsible for over 90% of the total protease activity in the extract (Warner, 1987; see Introduction). This observation suggests that the thiol protease regulation is extremely important in Artemia embryos. Why multiple forms of its inhibitor are present and why the inhibitors themselves may be under some type of proteolytic attack remains to be determined.

The presence of an inactive protease (P3) which requires activation by the thiol protease to unmask its activity has been identified in extracts from dormant Artemia embryos (Warner, 1987). The activity of this enzyme is both time and temperature dependent. Activated P3 has a temperature optimum of 40-45^{o}C and a pH optimum of 7.5-8.0. Its elution position on gel filtration columns suggests that it has a molecular weight between 5.0 and 10.0 kDa. It is an extremely stable protease and is unique as an enzyme due to its very small size. Due to its small molecular weight and stability it is likely that P3 is present in TPI prepara-

tions from Sephadex G-50. However, it is unknown whether it would be activated in TPI preparations during incubation at pH 5.0. Further research on this enzyme is needed to determine whether it is involved in any way in the hydrolysis of the thiol protease inhibitors in dormant Artemia embryos.

Most intracellular thiol proteases in mammalian systems are localized in lysosomes and their inhibitors are localized in the cytosol fraction (Katunuma and Kominami, 1985). Two separate research groups have identified the Artemia protease as a cathepsin B-like protease (see Introduction) and found that its activity is located both in the lysosomal and cytosol fractions (Warner and Shridhar, 1980; Perona and Vallejo, 1982). However, the site of cellular localization depends on the nature of homogenization media, and therefore the exact localization should be determined by histochemical techniques. Furthermore, the level of enzyme activity in the cytosol may be underestimated due to its sequestration by its analogous inhibitor. The thiol protease inhibitor activity in extracts from dormant Artemia embryos has been found only in the cytosol fraction.

The cathepsin B-like protease from dormant Artemia embryos is a dominant enzyme. By hatching time in Artemia 50% of the yolk platelets have been utilized and proteolytic degradation of lipovitellin occurs simultaneously. The lysosomal and cytosolic cathepsin B-like protease degrades lipovitellin in vitro, in a similar pattern to that seen in vivo. This suggests a role for the enzyme in yolk utiliza-

tion in Artemia development (see Introduction). Furthermore, lysosomes have been implicated in yolk platelet degradation in sea urchin embryos (Schuel et al., 1975). Therefore, the cathepsin B-like protease found in the lysosomal fraction in extracts from Artemia may assume a similar role.

The Artemia cathepsin B-like protease has also been implicated in the control of protein synthesis (Yablonka-Reuveni and Warner, 1979). These researchers observed that extracts from dormant embryos possessed a depressed translational capacity in cell-free translation systems and the presence of fragments of elongation factor II (EF-2) in the same extracts. It was then found that the cathepsin B-like protease selectively hydrolyzes EF-2 into lower molecular weight fragments between pH 6.0-6.6. At this pH the thiol protease inhibitor was found to be resistant to hydrolysis by the protease, and able to control its activity. Thus, a change in the distribution of EF-2, and its hydrolysis by this thiol protease during early development, may be a mechanism that controls the amount of EF-2 at the site of protein synthesis.

Control of thiol protease activity in Artemia embryos may occur by various mechanisms such as the ionic environment, intracellular pH, compartmentalization and the activity of thiol protease inhibitors (Warner et al., 1987).

The pH of Artemia embryos during aerobic development is approximately 7.9 (Busa et al., 1982). At this pH the cathepsin B-like protease is inactive in vitro (Warner and Shridhar, 1980; Perona and Vallejo, 1982). This finding suggests that the cathepsin B-like protease may be stabilized by compartmentalization or binding to some factor(s). Potential regulatory factors include protection in lysosomes or yolk platelets, sequestration by the thiol protease inhibitors or attachment to the cytoskeleton (Warner, 1987; Perona and Vallejo, 1982). The thiol protease inhibitors may serve a protective function whereby thiol protease molecules that leak into the cytosol from lysosomes are inactivated.

The results presented in this thesis raise many questions about the nature and function of low molecular weight thiol protease inhibitors in dormant Artemia embryos. Clearly, more research is required to purify these inhibitors in bulk in order to characterize them in pure form. Western blot analysis of the TPI's at various developmental stages in Artemia will reveal the developmental profile of these inhibitors during Artemia development. These studies may reveal the developmental role of the low molecular weight thiol protease inhibitors in Artemia development.

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